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THE UNIVERSITY OF ALBERTA

NUCLEOSIDE TRANSPORT IN K562 HUMAN LEUKEMIA CELLS

by

HARALABIA BOLETI

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA
FALL 1991

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SUBMITTED BY HARALABIA BOLETI

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY.

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Date June 5, 1991

To my parents Georgia and Dimitrios for their love and support with my education

To my dear friend Vassilis Karanassios who helped me believe that this task was possible

ITHACA

When you start on your journey to Ithaca,
then pray that the road is long,
full of adventure, full of knowledge.
Do not fear the Lestrygonians
and the Cyclopes and the angry Poseidon.
You will never meet such as these on your path,
if your thoughts remain lofty, if a fine
emotion touches your body and your spirit.
You will never meet the Lestrygonians,
the Cyclopes and the fierce Poseidon,
if you do not carry them within your soul,
if your soul does not raise them up before you.

Then pray that the road is long.
That the summer mornings are many,
that you will enter ports seen for the first time
with such pleasure, with such joy!
Stop at Phoenician markets,
and purchase fine merchandise,
mother of pearl and corals, amber and ebony,
and pleasurable perfumes of all kinds,
buy as many pleasurable perfumes as you can;
visit hosts of Egyptian cities,
to learn and learn from those who have knowledge.

Always keep Ithaca fixed in your mind.
To arrive there is your ultimate goal.
But do not hurry the voyage at all.
It is better to let it last for long years;
and even to anchor at the isle when you are old,
rich with all that you have gained on the way,
not expecting that Ithaca will offer you riches.

Ithaca has given you the beautiful voyage.
Without her you would never have taken the road.
But she has nothing more to give you.

And if you find her poor, Ithaca has not defrauded you.
With the great wisdom you have gained, with so much
experience,
you must surely have understood by then what Ithacas mean.

Konstantinos Kavafis

ABSTRACT

Nitrobenzylthioinosine (NBMPR) is a tight-binding inhibitor of equilibrative transport (facilitated diffusion) of nucleosides. Nucleoside transport by facilitated diffusion can be distinguished as NBMPR-sensitive or NBMPR-insensitive on the basis of inhibition by NBMPR. Inhibition of the NBMPR-sensitive process correlates with binding of NBMPR to high-affinity sites in the plasma membrane. Equilibrium binding studies revealed that K562/4 cells express a large number of high affinity NBMPR binding sites ($4.8 \pm 0.9 \times 10^5$ sites/cell, $K_d = 0.3$ nM). The concentration-effect relationships for inhibition by NBMPR of adenosine, uridine and thymidine zero-*trans* influx were biphasic, suggesting the presence of nucleoside transport processes of both high ($IC_{50} = 0.4$ -1.0 nM) and low ($IC_{50} > 1.0$ μM) sensitivity to NBMPR, accounting for 80-90% and 10-20% of total nucleoside transport activity, respectively.

Utilization of formycin B (a non-metabolizable inosine analog) and D- and L-enantiomers of uridine demonstrated that the NBMPR-sensitive and insensitive processes were equilibrative and stereoselective. As well, both NBMPR-sensitive and insensitive processes functioned in the absence of a Na^+ gradient. Uridine and adenosine zero-*trans* influx exhibited saturation kinetics, with different kinetic parameters for NBMPR-sensitive and insensitive influx. The NBMPR-sensitive process had higher affinities and higher capacities than the NBMPR-insensitive process, with 5- and 3-fold higher K_m values for uridine and adenosine, respectively, and 3-fold higher V_{max} values for both nucleosides. The efficiency of permeability (π_{12}^{zt}) was much higher (10-15 fold) for the NBMPR-

sensitive process, suggesting that it is the major route of uptake at low, physiologically relevant concentrations of permeant. However, K562/4 cells could not be protected by NBMPR from the cytotoxic effects of tubercidin. When K562/4 cells were exposed to tubercidin in the presence of 10 μM NBMPR, only a 3.5 fold increase in the IC_{50} of tubercidin was observed. Treatment of K562/4 cells with hemin, which induced partial erythroid differentiation, resulted in small changes in adenosine transport activity, mainly characterized by the almost complete disappearence of NBMPR-insensitive activity and an increase in the NBMPR-sensitive activity.

Photolabelling of membranes from K562 cells and human erythrocytes with ^{3}H-NBMPR identified polypeptides that migrated on SDS electrophoretograms as broad bands with different relative mobilities (peak values were, respectively, 63 and 55 kDa). The two polypeptides migrated with similar relative mobilities after deglycosylation with N-glycosidase F. Thus, although K562 cells exhibit "erythrocyte-like" nucleoside transport activity, the K562 transporter polypeptide is more heavily glycosylated than the erythrocyte polypeptide and may also have differences in primary structure as suggested by partial proteolysis with V8 protease.

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TABLE OF CONTENTS

Page

CHAPTER I-INTRODUCTION

A. NUCLEOSIDES: PHYSIOLOGICAL AND PHARMACOLOGICAL ROLE

1) Physiological nucleosides 1
2) Therapeutic nucleosides 2
3) Nucleoside salvage 3

B. NUCLEOSIDE TRANSPORT

1) Uptake of nucleosides-a two step process 4
2) Nitrobenzylthioinosine an inhibitor of nucleoside trasport 5
3) Heterogeneity of the nucleoside transport process 7
4) Implications of nucleoside transport for nucleoside therapeutics 11

C. MOLECULAR PROPERTIES OF THE NBMPR-SENSITIVE NUCLEOSIDE TRANSPORTER

1) Human erythrocytes 13
2) Other cells. 15

D. REGULATION OF NUCLEOSIDE TRANSPORT ACTIVITY

1) Erythroid cells 16
2) Other cell types 17

E. K562 ERYTHROLEUKEMIA CELLS

1) Origin 18
2) Erythroid nature of K562 cells 17
3) Induction of erythroid features 20
4) Induction of megacaryocytic differentiation 21

5) Membrane transport in K562 cells 22

F. OBJECTIVES

CHAPTER II - MATERIALS AND METHODS

A. MATERIALS

1) Materials 25

B. CELL CULTURE

1) Cell lines 26
2) Determination of proliferation rates 29
3) Determination of cell viability 30
4) Determination of protein content 30
5) Induction of hemoglobin production 30

C. TRANSPORT AND BINDING MEASUREMENTS TO INTACT CELLS

1) Composition of solutions used for assays 34
2) Procedures for measurement of transport 35
3) Determination of concentration-effect relationships for inhibition of transport by NBMPR 39
4) Calculation of transport rates 39
5) Analysis of transport kinetics. 40
6) Equilibrium binding of ^{3}H-NBMPRby intact cells 42

D. STUDY OF THE NBMPR-BINDING POLYPEPTIDE

1) Preparation of membrane fractions 43
    i) K562/4 cells
    ii) Human erythrocytes
2) Binding of ^{3}H-NBMPR to membranes 45
3) Photoaffinity labelling of membranes with ^{3}H-NBMPR 46
4) SDS-polyacrylamide gel electrophoresis 47

5) Deglycosylation of membranes with N-glycosidase F 48
6) Proteolysis of membranes with V8 protease 48

CHAPTER III - FUNCTIONAL CHARACTERIZATION OF THE NUCLEOSIDE TRANSPORT PROCESS IN K562/4 CELLS.

A. OVERVIEW 50
B. RESULTS
1) Effectiveness of NBMPR and dilazep as stopping reagents in the assay of uridine and adenosine transport by K562/4 cells 53
2) Characterization of the inhibition of uridine, adenosine and thymidine influx by NBMPR 56
3) Equilibrium binding of ^{3}H-NBMPR by K562 cells 71
4) Kinetics of uridine and adenosine zero-*trans* influx 75
5) Formycin B uptake in K562/4 cells 103
6) Effect of Na$^+$ on uridine and adenosine NBMPR -insensitive transport 107
7) Stereoselectivity of nucleoside transport in K562/4 cells. 110
8) Modulation of tubercidin toxicity in K562/4 cells by NBMPR 114
C. DISCUSSION 121

CHAPTER IV - IDENTIFICATION OF AN NBMPR-BINDING POLYPEPTIDE IN K562/4 CELLS AND STRUCTURAL COMPARISON WITH THE NUCLEOSIDE TRANSPORTER OF HUMAN ERYTHROCYTES

A. OVERVIEW 123

B. RESULTS

1) Identification of the K562/4 NBMPR-binding polypeptide by photoaffinity labelling with ^{3}H-NBMPR 124

2) Comparison of the electophoretic migration profiles of the ^{3}H-NBMPR-labelled polypeptides of K562/4 cells and human erythrocytes 127

3) Deglycosylation of NBMPR-binding polypeptides of K562/4 cells and human erythrocytes 133

4) Peptide map analysis of the NBMPR binding polypeptides K562/4 cells and human erythrocytes 136

C. SUMMARY 143

CHAPTER V - CHANGES IN NUCLEOSIDE TRANSPORT ACTIVITY IN K562/4 CELLS UPON HEMIN TREATMENT

A. OVERVIEW 144

B. RESULTS

1) Effect of hemin treatment on proliferation and hemoglobin expression in K562/4 cells 149

2) Effect of hemin treatment on binding of NBMPR to K562/4 cells 152

3) Effect of hemin treatment on adenosine transport by K562/4 cells 153
4) Effect of hemin on cell-cycle progression and cell size of K562/4 cells. 161
C. SUMMARY 164

CHAPTER VI - DISCUSSION 166
BIBLIOGRAPHY 186

LIST OF TABLES

Page

I. IC_{50} values for inhibition of uridine, adenosine and thymidine zero-*trans* influx by NBMPR 70

II. NBMPR binding constants for different clones of K562 cells 75

III. Comparison of three analytical methods for derivation of kinetic parameters of zero-*trans* influx of uridine and adenosine measured in the absence of NBMPR 100

IVA. Kinetic parameters of uridine zero-*trans* influx 101

IVB. Kinetic parameters of adenosine zero-*trans* influx 102

V. NBMPR binding activity in K562/4 cells treated with hemin 156

VI. Effect of hemin treatment on 1 μM adenosine zero-*trans* influx by K562/4 cells 160

VII. Summary of kinetic parameters for the NBMPR -sensitive and NBMPR-insensitive processes of K562/4 cells 170

IIX. Comparison of NBMPR-sensitive nucleoside transport characteristics and some other features of K562 cells and human erythrocytes 175

LIST OF FIGURES

Page

1. Effect of NBMPR on uridine uptake 58
2. Effectiveness of ice-cold dilazep as a stopping reagent for assay of uridine transport 60
3. The use of cold dilazep as a stopping reagent for assay of adenosine transport 62
4. Representative time courses for uridine zero-*trans* influx 64

5A. Concentration-effect relationships for inhibition by NBMPR of uridine *zero-trans* influx 67

5B. Concentration-effect relationships for inhibition by NBMPR of thymidine *zero-trans* influx 68

5C. Concentration-effect relationships for inhibition by NBMPR of adenosine *zero-trans* influx 69

6. Site-specific binding of ^{3}H-NBMPR to K562/4 cells 73
7. Time courses of uridine zero-*trans* influx 82
8. Time courses of uridine zero-*trans* influx in the presence of 0.1 μM NBMPR 84
9. Time courses of adenosine zero-*trans* influx 86
10. Time courses of adenosine zero-*trans* influx in the presence of 0.5 μM NBMPR. 88

11A. "0" time uridine uptake in K562/4 cells as a function of uridine extracellular concentration 90

11B. "0" time adenosine uptake in K562/4 cells as a function of adenosine extracellular concentration 90

12A. Concentration dependance of uridine zero-*trans* influx 92

12B. Concentration dependance of adenosine zero-*trans* influx. 92
13. Uridine zero-*trans* influx kinetics 94
14. Adenosine zero-*trans* influx kinetics 96
15. Direct linear plot for ^{3}H-uridine zero-*trans* influx 98
16. Formycin B uptake in K562/4 cells 106
17. Effect of Na^+ on NBMPR-insensitive zero-*trans* influx of uridine and adenosine 109
18. Enantiomeric selectivity of uridine transport in K562/4 cells 113
19. Inhibition of growth of K562/4 cells by tubercidin 118
20. Effect of NBMPR against tubercidin-induced inhibition of K562/4 cell growth 120
21. Analysis of ^{3}H-NBMPR labelled K562/4 membranes by SDS-polyacrylamide gel electrophoresis 126
22. Analysis of ^{3}H-NBMPR photolabelled membranes from K562/4 cells and human erythrocytes by SDS-polyacrylamide gel electrophoresis. 129
23 Analysis of a mixture of photolaballed NBMPR-binding polypeptides from human erythrocyte and membranes on SDS-polyacrylamide gel electrophoresis 131
24. Effect of N-glycosidase F on the electrophoretic mobility of ^{3}H-NBMPR-labelled polypeptides from K562/4 cells and human erythrocytes 135
25. Effect of *Staphylococcus aureus* V8 protease on ^{3}H-NBMPR labelled polypeptides from K562/4 cells and human erythrocytes 138

26. Effect of V8 protease on the electrophoretic mobility of ^{3}H-NBMPR labelled polypeptides of K562/4 cells and human erythrocytes 140
27. Effect of hemin treatment on proliferation, viability and hemoglobin expression in K562/4 cells 151
28. Effect of hemin treatment on site-specific binding of ^{3}H-NBMPR to K562/4 cells 154
29. Effect of hemin treatment on adenosine transport by K562/4 cells 159
30 Effect of hemin treatment on the cell-cycle distribution of K562/4 cells 163

ABREVIATIONS AND DEFINITIONS

Ado	β-D-adenosine
AraC	1-β-D-arabinosylcytosine
B_{max}	maximum number of ligand binding sites per cell as determined from equilibrium binding data by mass law analysis
dilazep	3,4,5-trimethoxybenzoic diester with tetrahydro-1H-1,4-diazepine-1,4(5H)-dipropanol
dThd	β-D-2'-deoxythymidine
DMSO	dimethylsulfoxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
IC_{50}	concentration of drug(s) inhibiting the parameter measured in individual experiments
K_d	dissociation constant
K_m	substrate concentration for half maximal unidirectional flux
M.D.	mean deviation
NBMPR	nitrobenzylthioinosine; 6-[(4-nitrobenzyl)-thio]-9-β-D-ribofuranosylpurine
NBTGR	nitrobenzylthioguanosine; 2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine
NMG^+	N-methyl-D-glucammonium
PBS	phosphate buffered saline
PMSF	phenyl-methyl-sulfonate fluoride

S.D.	standard deviation
SDS	sodium dodecylsulfate
Tubercidin	7-deazadenosine; 4-amino-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine
Tris	Tris-(hydroxymethyl)aminomethane
Urd	uridine
uv	ultraviolet

CHAPTER I

INTRODUCTION

A. NUCLEOSIDES

i) *Physiological nucleosides*

As precursor molecules of nucleotides, nucleosides play key roles in nearly all biochemical processes. Nucleotides are precursors of DNA and RNA, intermediates in many biosyntheses, energy-providing molecules, components of coenzymes, and metabolic regulators.

Of the physiological nucleosides, only adenosine is known to have a regulatory function in physiological and metabolic events. Adenosine is associated with control of neural activity by its inhibition of neurotransmitter release (Deckert et al 1988, Dunwiddle 1985). Adenosine also regulates vascular blood flow, antagonizes the effects of catabolic hormones and promotes the action of insulin, is involved in platelet aggregation and lymphocyte differentiation and inhibits pyrimidine biosynthesis (Fox et al 1978, Newby 1984). Adenosine acts by regulating adenylate cyclase activity through interaction with two classes of receptors termed A1 and A2. A_2 receptors mediate stimulation and A_1 receptors mediate inhibition of adenylate cyclase. Adenosine has been termed a "local hormone" (Newby 1984). Although it is continuously produced by many tissues, such as vascular endothelium, heart, brain and kidney (Fox et al 1978), only trace amounts are present in extracellular fluids (Sovelli et al 1982) Removal of adenosine from circulation occurs by rapid uptake in many cell types, including erythrocytes, via transport systems and further metabolism by cellular adenosine deaminase and adenosine kinase (Plagemann et al 1985).

ii) *Therapeutic nucleosides*

Of various cytotoxic nucleosides with clinical application as antineoplastic agents, the best known is 1-β-D-arabinosylcytosine (araC), which is an essential part of the current treatment of acute myeloid leukemia (Hess & Zirkle 1980). The 2-fluoro derivative of 9-β-D arabinosyladenine (F-ara-A) is cytotoxic against L1210 and P388 mouse leukemias and solid tumors (Montgomery 1983). Other haloadenine nucleosides also have cytotoxic properties. Among them the 2-haloadenine, arabinosylnucleosides and 2'-deoxyribosyl nucleosides appear to have the necessary selectivity for neoplastic cells to be useful anticancer agents (Montgomery 1983).

Guanine acyclic nucleosides (e.g., acyclovir), bromovinyl-deoxyuridine and related compounds, and 2'-fluoro-substituted pyrimidine arabinosides have selective antiherpes activity (De Clercq 1984, Hirsch & Schooley 1983). 3'-Azido-3'-deoxythymidine (AZT) is currently the accepted therapy for acquired immunodeficiency syndrome (AIDS) and 2',3'-dideoxycytidine is being used in AIDS clinical trials as a single agent or in alternate combination with AZT. Dideoxynucleosides constitute the potentially most useful class of anti-HIV agents currently available (De Clercq 1987). The antiviral action of many of these nucleoside analogues is by interaction with well-defined viral enzymes.

A large number of nucleoside analogs amongst which are some of the therapeutic nucleosides mentioned above enter and exit cells via the same processes as physiological nucleosides.

iii) *Nucleoside salvage*

Mammals do not have a nutritional requirement for purines or pyrimidines since most tissues have the capacity to synthesize purine and pyrimidine nucleotides *de novo*. There are, however, certain tissues that lack the synthetic pathways for purines (Murray 1971). Tissues in which the *de novo* purine pathway appears to be lacking are bone marrow, (Lajtha & Vane 1958, Thomson & Maddy 1960), leukocytes (Scott 1962), blood platelets (Holmsen & Rosenberg 1968) and erythrocytes (Lowry & Williams 1960, Fontenelle & Henderson 1969). Any turnover of purine nucleotides in these cell types must result from the continual entry and release of exogenous purines, either from the diet or endogenous production by other tissues, primarily liver (Lajtha & Vane 1958, Pritchard et al 1970). Whether capable of *de novo* synthesis or not, most tissues appear to be able to utilize purine and pyrimidine nucleosides by salvage pathways.

Cultured cells also possess the *de novo* synthetic pathways (Wohlhueter & Plagemann 1980) for purine and pyrimidine nucleotides and show no nutritional requirement for preformed purines or pyrimidines. Although exogenous nucleosides do not increase growth rates of cultured cells (Marz et al 1977), they are, if available, utilized to apparent advantage, avoiding the more energy-requiring *de novo* pathways. For most nucleosides, the pathways of utilization consist of permeation, by simple diffusion or mediated processes, followed by phosphorylation by a family of nucleoside

The role of the salvage process, which was proposed for purines (Murray 1971), is to enable synthetic products of one tissue to be made available to other tissues or to allow reutilization of purines and pyrimidines produced by turnover of nucleic acids and nucleotides. It appears, at least for purines, that control mechanisms exist to regulate *de novo* synthesis, release and subsequent reutilization by the liver and uptake by the blood for distribution to other tissues. These processes probably function to control the levels of free nucleosides in the circulation, thus regulating their physiological effects. For example, the release of adenosine from tissues such as vascular endothelium and its degradation by erythrocytes (Fox et al 1978, Parks et al 1985) may control the concentration of adenosine in the bloodstream.

B. NUCLEOSIDE TRANSPORT

In most cell types, functional nucleoside transport systems are probably required for the salvage of physiological purine and pyrimidine nucleosides for incorporation into cellular nucleotides and subsequent polymerization into nucleic acids. Nucleoside transport processes are also critical determinants of the pharmacologic action of nucleoside analogs with antineoplastic (Montgomery 1983), antiviral (Hirsch & Schooley 1983, De Clercq 1984) and antiparasitic activity (Suhadolnik 1979, El Kouni et al 1983).

i) *Uptake of nucleosides: a two-step process*

Cellular incorporation (uptake) of exogenous nucleosides into mammalian cells may be described as a two-step process: the permeation of nucleosides across the cell membrane, followed by phosphorylation, yielding metabolites to which the cell membrane is virtually impermeable (Wohlhueter & Plagemann 1980). With certain nucleosides, the process becomes more complex since other reactions (e.g., deamination) may compete with phosphorylation (Plagemann & Woffendin 1987). The rates of incorporation of a number of nucleosides into total cellular material follow simple Michaelis-Menten kinetics, suggestive of a saturable rate-determining step in the overall process (Plagemann et al 1978). For many cell-permeant combinations, free nucleosides equilibrate rapidly and the slower phosphorylation of intracellular nucleosides determines the long-term rate of incorporation. The combination of rapid mixing/sampling techniques with the use of inhibitors to stop transport reactions has made it technically possible to characterize nucleoside transport in cultured mammalian cells without interference from metabolic events.

ii) *Nitrobenzylthioinosine, an inhibitor of nucleoside transport*

The availability of various potent inhibitors (Paterson et al 1983) has enabled exploration of nucleoside transport systems in many cell types. Nitrobenzylthioinosine (NBMPR), the best studied member of a family of S^6-substituted-thiopurine nucleosides, binds reversibly and with high affinity to sites associated with nucleoside transport systems. NBMPR has been a valuable molecule used to (i) explore the heterogeneity of the nucleoside transport process, since the sensitivity of nucleoside transport to inhibition by NBMPR differs in different cell types, and (ii) to quantitate the number of NBMPR-sensitive nucleoside transporters. NBMPR binds with high affinity (Kd

< 5 nM) to plasma membrane sites which, in human erythrocytes, have been shown to be functionally associated with proteins that facilitate nucleoside permeation (Kwong et al 1988).

Binding sites with high affinity for NBMPR have been identified in a variety of other cell types (Lauzon & Paterson 1977, Dagnino 1988, Paterson et al 1987, Jarvis et al 1982). An association of NBMPR-binding sites with NBMPR-sensitive transporter elements is indicated by the characteristics of a transport-defective derivative of cultured S49 mouse lymphoma cells, which have lost both transport and NBMPR-binding activities (Cass et al 1981), suggesting that these activities are associated with the same gene product. The characteristics of other mutants of S49 cells suggest that NBMPR binds to the transporter polypeptide at a site with determinants that are distinct, at least in part, from those of the permeant binding site (Aronow et al 1985). In a single-step selection for resistance to the effect of NBMPR in preventing thymidine rescue of methotrexate toxicity, a mutant clone was obtained that had acquired resistance to NBMPR inhibition and had lost 70-75% of NBMPR-binding sites, whereas another retained the same number of NBMPR binding sites despite its resistance to NBMPR.

Although occupancy of the NBMPR-binding sites correlates directly with inhibition of uridine transport in human erythrocytes (Cass et al 1974) and Vmax values of uridine influx in erythrocytes from different species are proportional to the number of NBMPR transport-inhibitory sites (Jarvis et al 1982), the relationship between site occupancy and inhibition of transport in most other cell types is more complex. For example, in HeLa cells concentrations of NBMPR sufficient to saturate the high-affinity sites (about 5 nM) do not completely block the transport process, inhibiting transport activity no more than 70-75% (Dahlig-Harley et al 1981). In Novikoff rat hepatoma cells, micromolar concentrations of NBMPR are required for partial inhibition of nucleoside transport (Plagemann & Wohlhueter 1985), and NBMPR-binding sites are absent in some Novikoff cell lines and present in others (Gati et al 1986). Walker 256 rat mammary carcinoma cells, which possess a nucleoside transport process that is not inhibited by concentrations of NBMPR in the µM range, lack NBMPR-binding sites altogether (Paterson et al 1983).

iii) *Heterogeneity of the nucleoside transport process*

It is now well accepted that the sensitivity of nucleoside transport to inhibition by NBMPR varies in different cell types, and, for many, the relationship between transport inhibition and NBMPR concentration is biphasic, suggesting the operation of multiple transport systems. The terms "NBMPR-sensitive" and "NBMPR-insensitive" have been widely employed to describe systems that exhibit, respectively, high ($IC_{50} < 5$ nM) or low ($IC_{50} > 1$ µM) sensitivity to NBMPR inhibition. NBMPR-sensitive nucleoside transport is an equilibrative process termed facilitated diffusion. This process has been extensively characterized in human erythrocytes and fulfills the criteria of a simple carrier model (Lieb & Stein 1976). Transport systems of this nature are widely distributed (Gati & Paterson 1989). NBMPR-insensitive nucleoside transport has been found in some cell types to be a facilitated diffusion process (Belt & Noel 1985, Plagemann et al 1978) and in others to be a Na^+-dependent, concentrative process (Jarvis 1989, Williams et al 1989, Plagemann & Woffendin 1989, Vijayalakshmi & Belt 1988).

Equilibrative nucleoside transport processes that are insensitive to NBMPR have been partially characterized in two neoplastic rat cell lines, Novikoff hepatoma and Walker 256 mammary carcinoma (Plagemann & Wohlhueter 1985, Belt and Noel 1985). Nucleoside permeation processess of low NBMPR sensitivity have also been recognized in other cell lines (Belt 1983, Plagemann & Wohlhueter 1984, Jarvis & Young 1986). NBMPR-insensitive and NBMPR-sensitive equilibrative systems are genetically separable (Aronow et al 1984, Plagemann & Woffendin 1987) and cells that have NBMPR-insensitive transport may or may not possess sites that bind NBMPR with high affinity.

Na^+-dependent, concentrative, nucleoside influx systems have been identified in brush border membranes of epithelial cells of rat, mouse and rabbit kidney and intestine, in mouse spleen cells and in L1210 mouse leukemia and HL60 human promyelocytic leukemia cells (Darnowski et al 1987, Plagemann & Woffendin 1989, Jarvis 1989, Vijayalakshmi & Belt 1988, Dagnino 1988, Lee et al 1988, Lee et al 1990). The permeant specificities of these systems differ with the cell type (Darnowski et al 1987, Vijayalakshmi & Belt 1988), and the systems identified thus far are insensitive to inhibition by NBMPR.

Channel-like nucleoside permeation has been recognized in cultured human ovarian carcinoma cells (Adjei 1989) and in erythrocytes infected with malarial parasites (Gati et al 1987). In cells in which this process has been characterized, nucleoside fluxes are unsaturable, NBMPR insensitive, and nonselective with respect to the D- and L-enantiomers.

For the physiologic nucleosides and probably most nucleoside analogs, non-mediated permeation (simple diffusion) constitutes a minor component of total nucleoside permeation. This is evident by the low permeation rates reported for NBMPR-treated S49 mouse lymphoma cells, for nucleoside-transport deficient mutants derived from S49 cells (Cass et al 1981), and for L-adenosine in erythrocytes and L1210 mouse leukemia cells (Gati et al 1989).

While human erythrocytes express only the equilibrative, NBMPR-sensitive system, several other cell types express more than one nucleoside transport processes. For example cultured mouse leukemia L1210 cells express at least three distinct systems: an NBMPR-sensitive equilibrative system, an NBMPR-insensitive equilibrative system and a Na^+-dependent, concentrative system (Dagnino 1988, Hogue et al 1990). IEC-6 rat intestinal epithelial cells coexpress both sodium-dependent NBMPR-insensitive transport and equilibrative NBMPR-sensitive transport activities (Jakobs et al 1991).

iv) *Nucleoside transport in human erythrocytes*

Initial observations that a single transport system facilitates permeation of uridine and other nucleosides across the plasma membrane of erythrocytes (Oliver & Paterson 1971, Cass 1972) were followed by extensive studies of the kinetics of uridine transport that examined influx and efflux of uridine under equilibrium-exchange, zero-*trans* and infinite-*cis* conditions (Cabantchic 1977). These and later studies provided evidence that uridine movement across erythrocyte membranes can be described by a simple carrier mechanism that displays asymmetrical properties in stored erythrocytes and symmetrical properties in fresh erythrocytes (Jarvis et al 1983, Plagemann 1984, Cabantchic 1977). Relationships between transport rates and substrate concentrations can be described by the Michaelis-Menten equation. The nucleoside transporter of erythrocytes exhibits differential mobilities in the loaded and empty states, as indicated by the greater maximum velocity of equilibrium exchange diffusion (V^{ee}) than of zero-*trans* flux (V^{zt}) of uridine (Jarvis et al 1983).

Like many other transport processes of erythrocytes, nucleoside transport displays a high activation energy. The temperature dependence of equilibrium exchange diffusion of uridine was determined in a study with human erythrocytes (Plagemann & Wohlhueter 1984). The maximum velocity increased in a linear fashion as the temperature was raised from 5° to 30°C ($E\alpha$=17-20 Kcal/mol) and then leveled off above 30°C.

The nucleoside transporter of human erythrocytes accepts as substrates nucleosides that differ widely in the base moiety but certain modifications in the ribofuranosyl moiety are not tolerated (Cass & Paterson 1972, Gati et al 1984). The 2'- and 3'-OH groups play an important role for transport. The kinetic parameters for 2'-deoxyadenosine transport are similar to those for adenosine transport whereas the lack of the 3'-OH group greatly reduces the affinity of 3'-deoxyadenosine for the carrier. In addition, transport of 2',3'-dideoxynucleosides is only 1% as efficient as that of 2' or 3'-deoxynucleosides (Plagemann et al 1990). The mobility of the carrier when loaded with different nucleosides is inversely related to the lipid solubility of the transported nucleoside, as estimated by its solubility in octanol (Plagemann et al 1990).

The enantiomeric configuration of the ribosyl moiety is a determinant of interaction of nucleosides with the transport systems of human (Gati et al 1991) and mouse (Gati et al 1989) erythrocytes, and cultured L1210/AM cells (Gati et al 1989) and S49 cells (Gati et al 1990). The natural isomers, D-uridine and D-adenosine, are preferred as substrates over the L-enantiomers (Gati et al 1989, 1991).

v) *Implications of nucleoside transport for nucleoside therapeutics*

The diversity of nucleoside transport processes has led to the idea that selective action of nucleoside drugs against target cells may be influenced by transport inhibitors, such as NBMPR or dipyridamole, a potent non-nucleoside inhibitor of transport (Paterson et al 1983). Research up to now has aimed at using transport inhibitors to enhance the therapeutic activity of nucleoside drugs to tumor cells by (i) increasing selectivity of drug uptake and/or retention in target cells, and (ii) reducing drug uptake by non-target cells (Paterson et al 1987, Kapilnsky et al 1986).

Naturally occurring differences in sensitivity of various cell types to NBMPR, or related inhibitors, has been suggested as the basis for development of new chemotherapeutic combinations in cancer treatment (Paterson et al 1983). The rational assumes an ability to selectively protect dose-limiting host tissues. In the presence of NBMPR, cancer cells with NBMPR-insensitive processes would be more susceptible to toxic nucleosides than vital host cells with NBMPR-sensitive processes. This approach has been used successfully in the treatment of rats bearing an NBMPR-insensitive tumor, the Walker 256 carcinosarcoma, with tubercidin, a nucleoside with significant host toxicity. When tumor-bearing rats were treated with tubercidin together with the 5'-monophosphate of NBMPR, long-term survival was achieved, whereas none of the animals treated with either agent alone survived (Paterson et al 1987). Enhancement of toxicity has also been observed with a few other combinations of nucleosides and transport inhibitors (Paterson et al 1983).

Combination protocols, involving the 5'-phosphate of NBMPR and tubercidin, have been utilized successfully in treatment of mice with shistosomiasis (El Kouni et al 1983). In addition, when mice infected with *Plasmodium yoellii* were treated with combinations of tubercidin and NBMPR phosphate, progression of parasitemia was delayed and survival times were increased (Gati et al 1987).

Clinical use of nucleoside transport inhibitors has been suggested as a means to increase extracellular levels of endogenous adenosine, thus enhancing its physiological role. Transport inhibitors could have beneficial therapeutic effects in a variety of CNS disorders, ranging from problems with sleep and regulation of autonomic functions like respiration and blood pressure, to anxiety and seizures (Deckert et al 1988). Marked decreases in the rates of adenosine degradation by erythrocytes through inhibition of nucleoside transport may play a key role in the antithrombotic effects of antiplatelet drugs such as dipyridamole and dilazep (Parks 1985).

C. MOLECULAR PROPERTIES OF THE NBMPR-SENSITIVE NUCLEOSIDE TRANSPORTER

NBMPR has been a powerful probe for exploring the molecular properties of proteins involved in nucleoside transport. This is due to (i) its ability to bind tightly to membrane sites that are believed to be an integral part of nucleoside transporter polypeptides in erythrocytes and various other cell types, and (ii) its photoreactivity. Upon exposure to uv light, site-bound ^{3}H-NBMPR crosslinks to its binding site (Young et al 1983). The mechanism of covalent attachment of ^{3}H-NBMPR to transporter polypeptides is not very clear, however experimental evidence suggests that NBMPR itself forms the reactive species (Jarvis & Young 1987). The primary photoreactive site on NBMPR is the sulfur-benzyl bond (Fleming et al 1990). Although N^6-(p-azidobenzyl)adenosine (Young et al 1983) and ^{3}H-dipyridamole (Woffendin & Plagemann 1987) have also been employed as covalent probes of nucleoside transporter polypeptides of human erythrocytes, NBMPR has been the most frequently used ligand in photolabelling studies.

i) *Human erythrocytes*

Exposure of human erythrocyte membranes to uv light in the presence of specifically bound ^{3}H-NBMPR results in selective incorporation of radioactivity into band 4.5 polypeptides. Band 4.5 polypeptides nomenclature of (Steck 1974) migrate on SDS-polyacrylamide gels as a heterogenous mixture, with Mr values of 45-66 kDa.

A limitation in efforts to isolate and characterize the nucleoside transporter from human erythrocyte membranes has been that only a small fraction of total membrane protein is involved in nucleoside translocation. The 10^4 nucleoside transport elements in each human erythrocyte represent approximately 0.1 % of total membrane protein, assuming an equivalence between the NBMPR-binding protein and the transporter protein (Young et al 1983, Jarvis & Young 1981). Another limitation has been the apparent similarities in structural properties of the glucose and nucleoside transporters. In human erythrocytes, these proteins comigrate in the band 4.5 region of SDS-polyacrylamide gels with an identical apparent molecular mass of 55 kDa and copurify in DEAE-cellulose ion-exchange chromatography. Purified band 4.5 preparations from human erythrocytes consist mainly of the glucose transporter (about 95 %) with the nucleoside transporter present as a minor component ($\leq$ 5 %). The glucose transporter is a more abundant species (3 x 10^5 copies/cell) than the nucleoside transporter.

The glucose and nucleoside transporters of human erythrocytes are distinct molecules. Different patterns have been observed when membranes photolabelled with ^{3}H-NBMPR or ^{3}H-cytochalasin B were subjected to peptide map analysis (Klip & Walker 1986) or *in situ* digestion for investigation of transmembrane topology (Janmohamed et al 1985). Genetic evidence also suggests that the nucleoside and glucose transporters are separate gene products. A mutant mouse lymphoma cell line was selected that had lost the capacity to transport thymidine and bind NBMPR but retained the glucose transport function (Klip & Walker 1986).

The nucleoside transporter of human erythrocytes has been purified (Kwong et al 1988) to near homogeneity by passage of band 4.5 preparations through an affinity column of immobilized antibodies specific for the glucose transporter. With this procedure, the glucose transporter was retained on the column and the nucleoside transporter was subsequently purified from the flow-through fraction. The isolated protein migrated as a single broad band of apparent Mr 55,000 in SDS-polyacrylamide gel electrophoresis, bound about 0.6 mol of NBMPR/mol of polypeptide with a Kd of 1.1 ± 0.14 nM and was not labelled in Western blots by monoclonal antibodies raised against the human erythrocyte glucose transporter. The identity of the protein was confirmed by reconstitution of uridine transport and NBMPR-binding activities into large unilamellar phospholipid vesicles. The reconstitution experiments with purified NBMPR-binding protein established that the nucleoside transport protein is distinct from the glucose transporter protein, since the reconstituted liposomes did not have glucose transport activity.

ii) *Other cells.*

NBMPR-binding polypeptides have been identified by photoaffinity labelling in a variety of cell types of different species (Gati & Paterson 1989a). In some cell types, the photolabelled polypeptides are comparable in size to the human erythrocyte polypeptide (50-56 kDa) while in others the photolabelled polypeptides are significantly larger (62-87 kDa). For rat erythrocytes, CCRF-CEM human T-cell lymphoma cells and rat liver (Crawford et al 1990, Jarvis 1986), these differences in molecular mass can be abolished by treatment with carbohydrate-removing enzymes, whereas for pig erythrocytes, differences are maintained even after deglycosylation (Kwong et al 1986, Craik et al 1988).

In CCRF-CEM cells (Crawford et al 1990), the ^{3}H-NBMPR-labelled polypeptides migrated in SDS-polyacrylamide gel electrophoresis as a broad band with an apparent molecular mass (75 ± 3 kDa) that was significantly higher than that reported for the nucleoside transporter of human erythrocytes (55 kDa). When membranes from mutant CEM cells that are deficient in nucleoside transport were photolabelled with ^{3}H-NBMPR, there was no specific incorporation of radioactivity into membrane fractions analyzed by SDS-polyacrylamide gel electrophoresis. Reconstitution in phospholipid vesicles of CEM membrane fractions enriched in NBMPR-binding activity resulted in reconstitution of uridine transport activity that could be inhibited by NBMPR.

D. REGULATION OF NUCLEOSIDE TRANSPORT ACTIVITY

i) *Erythroid cells*

There is some evidence that nucleoside transport activity may be subject to regulation during erythroid maturation. Culture techniques, which enable reticulocyte maturation to be followed *in vitro*, have been used to study changes in plasma membrane structure and function associated with maturation of reticulocytes to erythrocytes (Tucker & Young 1980, Weigensberg & Blostein 1983). Although reticulocytes from sheep and guinea pigs have high nucleoside-transport activities, maturation during *in vitro* culture results in the parallel loss of transport and NBMPR-binding activities

(Jarvis & Young 1982). The mechanism responsible for the regulation of membrane transport during reticulocyte maturation is not known.

The only cultured cells of erythroid nature in which nucleoside transport has been examined are Friend murine erythroleukemia cells. In these cells, uridine fluxes ($V_{max}/\mu m^2$ of cell surface area) remained unchanged upon induction to erythroid differentiation with dimethylsulfoxide (DMSO) (Gordon & Rubin 1982). However, transport rates were estimated in this study from uptake courses obtained over 10-15 sec exposure of cells to ^{3}H-uridine. Since the transport assays were performed at 37°C, 10-15 sec uptake courses were sufficiently long for intracellular metabolism to occur and thus the described observations may not represent only changes in transport.

ii) *Other cell types*

Nucleoside transport changes have also been observed in the course of differentiation of various non-erythroid cells. A decrease in nucleoside transport activity occured during N,N'-dimethylformamide or DMSO-induced granulocytic maturation of HL-60 human promyelocytic leukemia cells. The reduction in nucleoside transport capacity after N,N'-dimethylformamide-induced differentiation of cultured HL-60 cells was accompanied by a 95% decrease in the number (sites/cell) of specific NBMPR-binding sites (Chen et al 1986). AraC transport activity decreased during 12-0-tetradecanoyl-phorbol-13 acetate (TPA)-induced differentiation of the human T-lymphoblastoid cell line MOLT 4 along the T-lineage (Takimoto et al 1988). In freshly isolated peripheral blood lymphocytes from humans, araC influx and NBMPR-binding activity for leukemic myeloblasts and lymphoblasts were 5- and 3-fold higher than for mature cells of the same lineages (Wiley et al 1982).

Induction of Na^+-linked nucleoside transport activity in IEC-6 intestinal epithelial cells (Jakobs et al 1991) has been associated with the attainment of cell confluence and cessation of proliferation. Expression of Na^+-linked nucleoside transport activity was strongly affected by the composition of the growth medium, and growth factors (EGF, insulin and hydrocortisone) enhanced the expression of Na^+-linked transport activity.

D. K562 ERYTHROLEUKEMIA CELLS

i) *Origin*

The K562 cell line was established in 1975 (Lozzio & Lozio 1975) from cells in pleural effusion fluids of a patient with chronic myeloid leukemia (CML) undergoing blast crisis. K562 cells carry the Philadelphia chromosome (Ph^1), an abnormality in which a portion of the long arm of chromosome 22 has been translocated to chromosome 9. The translocation, which is found in leukemic cells of 80-90 % of CML patients, links the *c-abl* protooncogene (Heisterkamp et al 1983) on chromosome 9 to a break point cluster region (*bcr*) on chromosome 22 (Groffen et al 1984), resulting in production of a hybrid mRNA containing both *bcr* and *c-abl* sequences (Grosveld et al 1986).

ii) *Erythroid nature of K562 cells*

K562 cells are large (15-20 µm in diameter), with a high nucleus-to-cytoplasmic ratio and an irregular surface with ruffled membranes (Klein et al 1976). The cell line was originally considered to be of granulocytic origin because of the presence of granulocytic antigens (Drew et al 1977) and lysozyme (Greenberger et al 1978) and the absence of T and B cell markers (Lozzio et al 1976, Lozzio & Lozio 1979). However, subsequent observations demonstrated both constitutive and induced expression of a number of erythroid characteristics by K562 cells.

The spectrum of surface glycoproteins of K562 cells, identified by galactose oxidase-NaB[^{3}H]$_4$ labelling and gel electrophoresis, is similar to that of normal erythrocytes, but different from that of normal and malignant cells of the granulocytic lineage (Anderson et al 1979a). K562 cells (Gahmberg et al 1979) express glycophorin A, the major sialoglycoprotein on human erythrocytes expressed by differentiating erythroid cells in normal human bone marrow before the onset of detectable hemoglobin synthesis (Gahmberg et al 1978). Spectrin, a major extrinsic membrane protein of erythrocytes, has been identified in K562 cells by immunofluorescence with antibody against spectrin (Marie et al 1981). Acetylcholinesterase, a membrane-bound enzyme that is considered a marker of erythroid commitment, is also present in K562 cells (Ajmar et al 1983, Garre' et al 1984).

Although the K562 line was established from leukemic cells from an adult patient, many of the erythroid characteristics exhibited by K562 cells are those of embryonic or fetal, rather than adult, hematopoietic cells (Benz et al 1980). For example, i surface antigen (a linear surface carbohydrate) and lactate dehydrogenase isoenzymes found in K562 cells are characteristic of embryonic or fetal erythroid cells. K562 cells also express constitutively low levels of fetal and embryonic globin and globin mRNA (Benz et al 1980).

The erythroid nature of K562 cells is also demonstrated by their responsiveness to erythroid-specific growth factors. K562 cells produce erythroid potentiating activity (EPA) and also possess specific cell surface receptors for EPA (Avalos et al 1988). A low number of high-affinity surface receptors for erythropoietin are present on K562 cell surfaces (Frazer et al 1988). Exposure of K562 cells to conditioned medium from a cell line that secretes EPA or to recombinant EPA increases expression of receptors for erythropoietin by 3-fold (Fraser et al 1988).

ii) Induction of Erythroid Features in K562 Cells

An increase in expression of erythroid characteristics results when K562 cells are exposed to a variety of agents, including sodium butyrate, hemin and several anticancer drugs. Treatment of K562 cells with sodium butyrate, which induces differentiative responses in various other cultured cell lines (Ginsburg et al 1973, Reiss et al 1986), stimulates, in reversible fashion, production of hemoglobin, as detected by benzidine staining and radioimmunoassay (Anderson et al 1979b). Exposure of K562 cells, growing in semisolid agar, to sodium butyrate gives rise to benzidine-positive colonies that resemble erythroid colonies derived from normal erythroid stem cells. Addition of erythropoietin to such cultures synergistically enhances formation of erythroid colonies (Hoffman et al 1979).

Rutherford et al (1980) has established that embryonic hemoglobin and small quantities of fetal hemoglobin are produced after exposure of K562 cells in suspension culture to hemin. Induction of hemoglobin expression by treatment of K562 cells with hemin is reversible (Dean et al 1981). The electrophoretic pattern of the hemoglobins from K562 cells corresponds closely with that observed for hemoglobins from embryonic erythroblasts. The levels of glycophorin, i antigen, embryonic globin and embryonic globin mRNA increase substantially after exposure of K562 cells to hemin (Sutherland 1984, Benz et al 1980).

Other agents also induce expression of erythroid characteristics in K562 cells. Exposure to araC, an inhibitor of DNA polymerase used in therapy of leukemia, causes a coordinate irreversible increase in expression of hemoglobin and acetylcholinesterase, an effect associated with the loss of the ability of cells to form colonies. Exposure of K562 cells to anthracycline antibiotics (Trentesaux et al 1989, Toffoli et al 1989) also causes an irreversible induction of hemoglobin synthesis.

iv) Induction of Megacaryocytic Features

Although the erythroid nature of K562 cells is well documented, a number of reports indicate that K562 cells also have the potential to express megakaryocytic characteristics (Tabillio et al 1984, Teteroo et al 1984). Treatment of K562 cells with the tumor-promoting phorbol ester TPA causes an increase in megacaryocytespecific antigens and disappearance of glycophorin (Siebert & Fukuda 1985), increase in cell size and induction of synthesis of platelet derived growth factor (PDGF) (Colamonici et al 1986, Alitalo et al 1987), and enhanced expression of transforming growth factor (TGF)-β mRNA (Alitalo et al 1988). Na butyrate, which induces expression of erythroid characteristics by K562 cells, also induces expression of some megakaryocytic characteristics (Vainchenker et al 1984).

Cells of the K562 line are thought by many authors to represent a spectrum of pluripotential cells with different capacities for differentiation toward the erythroid and megakaryocytic cell lineages. Although some granulocytic markers are expressed by K562 cells, attempts to obtain granulocytic differentiation have failed (Vainchenker et al 1984).

v) *Membrane Transport in K562 cells*

Very little is known about membrane transport in K562 cells. In a comparison of adenosine uptake by K562 cells and human erythrocytes (Muller et al 1983), K562 cells accumulated radiolabel from adenosine to a much greater extent than did erythrocytes. The more rapid uptake by K562 cells was attributed to high levels of phosphorylation since K562 cells exhibited 10-fold higher levels of adenosine kinase activity than erythrocytes. Uptake of purine bases was also greater in K562 cells than in erythrocytes (Muller et al 1983).

Although results of a kinetic study of 3-O-methylglucose transport suggested that K562 cells possess a glucose transporter similar to that of human erythrocytes, there was a large difference between the two cell types in transport capacity (Dozier et al 1981).

The Vmax (pmol/μl cell water/min) for transport of 3-O-methylglucose by K562 cells was 100-fold higher than that for erythrocytes. The glucose transporter of K562 cells appears to be homologous to the HepG2 erythrocytic glucose transporter, since mRNA species recognized by HepG2 "erythroid/brain" cDNA were detected in K562 cells (Mueckler et al 1988).

The anion exchange properties of K562 cells differ markedly from those of mature human erythrocytes. Exchange of Cl^- in K562 cells is considerably less than that of mature human erythocytes (Law et al 1983). In addition, K562 cells exhibit low selectivity for Cl^- and SO_4^{-2}, and various agents that inhibit Cl^- exchange in erythrocytes are much less effective in K562 cells. Anion transport properties are unchanged by treatment of K562 cells with agents that induce hemoglobin synthesis (Law et al 1983). K562 cells express a surface glycoprotein of about 105 kDa (Fukuda 1980, Horton et al 1981), which resembles the fetal form of band 3 in that it possesses a linear form of the large oligosaccharide chain known as erythroglycan (Turco et al 1980). The 105 kDa glycoprotein differs from band 3 of mature erythrocytes since it was not labelled with $[^3H]$-H_2DIDS (4,4'-diisothiocyano-dihydrostilbene-2,2'-disulfonate), a probe that selectively reacts with the erythroid band 3 (Law et al 1983), and was not precipitated by antibodies against the erythroid band 3 (Fukuda 1980). The above results suggesting the expression of a non-erythroid band 3 protein in K562 cells were confirmed by detection of mRNA species encoding a protein that is similar in sequence but not identical to the human erythroid band 3 (Demuth et al 1986).

E. OBJECTIVES

Although the NBMPR-sensitive nucleoside transport process has been well characterized in erythrocytes and many cultured cell lines, nothing is known about regulation of expression of the proteins that mediate transport. This work was undertaken to identify a cultured cell line that possesses an NBMPR-sensitive transporter similar to that of human erythrocytes that could be used, when molecular and immunologic probes for the erythrocyte transporter polypeptide become available, to study biosynthesis and regulation of expression of the erythrocyte-like nucleoside transporter.

The K562 cell line expresses characteristics of erythroid origin and its biological properties have been extensively studied. The K562 cell line was selected for this work because, being derived from human myeloid leukemia, it was expected to express the erythrocyte-like nucleoside transporter. K562 cells constitutively express a number of features characteristic of adult and/or fetal erythrocytes and respond to treatment with differentiation-inducing agents by up-regulating expression of markers of both erythroid and megakaryocytic differentiation. Because of its differentiative response, the K562 cell line is a potentially useful model for studies of developmentally regulated changes in nucleoside transport.

The specific objectives of this work were:

(1) to determine if K562 cells exhibited mediated transport of nucleosides and, if so, to determine which nucleoside transport systems were present,

(2) to characterize the kinetics of the transport systems identified in K562/4 cells,

(3) if equilibrative NBMPR-sensitive transport activity was identified, to characterize interaction of NBMPR with K562/4 cells by mass law analysis and photolabelling of membrane polypeptides,

(4) to compare the structural features of NBMPR-binding polypeptides of K562/4 cells, if present, with those of human erythrocytes, and

(5) to determine if induction of the erythroid phenotype in K562/4 cells resulted in changes in nucleoside transport.

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

Cell culture materials and gentamicin were purchased from GIBCO (Burlington, Ont.) and the mycoplasma contamination testing kit from GEN-PROBE (San Diego, CA). Dilazep dihydrochloride was a generous gift from F. Hoffman La Roche & Co. (Basel, Switzerland). Paraffin oil (Saybott viscosity 125-135) and silicone 550 oil were from Fisher Scientific (Ottawa, Ont.) and Dow Corning (Mississauga, Ont.), respectively. Percoll and density-marker beads were obtained from Pharmacia (Dorval, Que.). L-Uridine (Gati et al 1991) and NBMPR (Paul et al 1975) were kindly provided, respectively, by Drs. W.P. Gati and A.R.P. Paterson (Department of Pharmacology University of Alberta). Uridine, thymidine, adenosine, tubercidin, hemin (equine type III), 3,3',5,5'-tetramethyl benzidine dihydrochloride (in the form of 1 mg tablets) and hemoglobin were purchased from Sigma Chemical Company (St. Louis, MO). Materials for electrophoresis were purchased from Bio-Rad Laboratories (Mississauga, Ont.) and Econofluor, Protosol and Enlightening solutions were from New England Nuclear (Boston, MA). Other chemicals were obtained from standard commercial sources.

Peptide-N-glycosidase F (N-glycanase) was purchased from Genzyme Corp. (Boston, MA), Glu-C endoproteinase (*Staphylococus aureus* V8) was from Boehringer Mannheim Canada (Laval, Que.) and trypsin and chymotrypsin were from Sigma Chemical Company (St. Louis, MO).

[5,6-^{3}H]Uridine (50 Ci/mmol), [methyl-^{3}H]thymidine (60 Ci/mmol), and [2,8-^{3}H]adenosine (32 Ci/mmol) were obtained from

ICN Radiochemicals, Inc. (St. Laurent, Que.). $[^3H]H_2O$ (1 mCi/ml), $[^{14}C(U)]$sucrose (671 mCi/mmol), $[^3H(G)]$NBMPR (23 Ci/mmol), L-[5-^3H]uridine (24 Ci/mmol) and $[^3H(G)]$formycin B (1.5 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA).

^3H-Labelled nucleosides were purified by high-pressure liquid chromatography (HPLC) using a Spectra Physics 8000A instrument equipped with a C18 reverse-phase column (Partisil 10 ODS-3 column, Whatman Inc Clifton, NJ). $[^3H(G)]$-NBMPR was purified by isocratic elution with 50% methanol in water. [5,6-^3H]Uridine, [methyl-^3H]thymidine, [2,8-^3H]adenosine, L-[5-^3H]uridine and $[^3H(G)]$formycin B were purified by elution with methanol-water gradients (0-20% methanol for thymidine and 0-30% for all other nucleosides; 35 min at 2 ml/min). Retention times (expressed in min) were: adenosine (25.4-27.6), uridine (14.3-15.9), L-uridine (12.5-15.7), thymidine (21.5-26.7), formycin B (17.3-19.9) and NBMPR (14.9-16.9). Repurified nucleosides were stored in methanol/water solutions at 4°C and were used within 2 months.

B. CELL CULTURE

1) Cell lines

i) *Origin and characteristics of K562 cells*

The K562 cell line was established *in vitro* from a pleural effusion of a patient with CML in terminal blast crisis (Lozzio & Lozzio 1975) and was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). K562/4, the subline used for most of the work in this study, is a cloned derivative of K562(ATCC) cells and was obtained from Dr. P. Manoni (Department of Pathology, University of

Alberta). The origin and characteristics of K562/4 cells have been described (Sutherland 1984). When actively proliferating, K562/4 cells are morphologically similar to primitive blast cells and express low levels of hemoglobin, glycophorin A and early granulocytic markers (Sutherland 1984). The K562/1A3 and K562/2B1 sublines are also clonal derivatives of the ATCC line and were obtained as follows. Actively proliferating cultures of K562(ATCC) were diluted to a concentration of 10 cells/ml and 0.2-ml portions were distributed in 96-well microtiter plates. After 7-10 days, cells that proliferated and gave rise to colonies were selected, expanded and subjected to a second round of cloning by limited dilution. K562/1A3 and K562/2B1 cells exhibited proliferation rates that were similar to those of the parental cells and were selected for further study.

ii) *Maintenance of K562 cultures*

All cell lines were grown at 37°C as suspension cultures in RPMI 1640 medium supplemented with 5% fetal bovine serum and 5% calf serum. Stock cultures were free of mycoplasma contamination. Mycoplasma testing (GEN-PROBE mycoplasma tissue culture rapid detection system) was conducted by in-solution hybridization, according to the manufacturer's instructions using a ^{3}H-labelled DNA probe, which is homologous to *Mycoplasm*a and *Acholeplasma* ribosomal RNA, and thus detects the species of mycoplasma commonly found in cell cultures. Mycoplasma-negative cultures were frozen in growth media with 10% dimethylsulfoxide and stored in liquid nitrogen. Cultures started from the frozen stocks were passaged for up to 30 culture generations and then discarded.

When cultures were expanded for experiments, 50 μg/ml gentamicin was added to growth media. Although stock cultures were also sometimes grown in media that contained gentamicin, those that were subsequently frozen for storage were always grown in the absence of antibiotic. There were no effects on either the growth characteristics or transport properties that could be attributed to gentamicin. The cells were maintained in 25-, 50-, or 100-ml cultures in a humidified 5% CO_2/air atmosphere and were diluted to 1.0 x 10^5 cells/ml with fresh growth media every 2-3 days. The cultures were actively proliferating when population densities were between 1 x 10^5 and 6 x 10^5 cells/ml.

To obtain the large numbers of cells required for transport studies, cultures were grown in 0.5-L or 1.0-L roller bottles gassed with 10% CO_2/air and turned by a Cell Production Roller Apparatus (Bellco Glass, Inc., Vineland, NJ) at 0.5 rpm. The roller cultures were initiated at 1 x 10^5 to 2 x 10^5 cells/ml and their volumes did not exceed 0.3-0.4 L and 0.7-0.8 L for the 0.5- and 1.0-L roller bottles, respectively. These cultures were actively proliferating when the population densities were between 1 x 10^5 to 9 x 10^5 cells/ml, and cells were harvested for experiments from cultures whose population densities did not exceed 6 x 10^5 cells/ml.

Cell numbers were determined using a Model Z_F electronic particle counter with a 100-μm aperture tube interfaced with a 100-channel particle size analyzer (Channelyzer II) and connected to an X-Y recorder for determination of cell size distributions. The particle size analyzer was calibrated with polystyrene microspheres of 9.63

μm diameter. The counting system and microspheres were from Coulter Electronics Inc., Oakville, Ont.

2) Determination of Proliferation rates

Cell proliferation rates are expressed as the number of population doublings that occurred in cultures during 24- or 48-hr intervals. Cell numbers were determined by electronic particle counting of duplicate 1-ml samples. The number of population doublings was estimated by subtracting the $\log_2$ of the initial population density at 0 hr from the $\log_2$ of the population density at 24 or 48 hr. For actively proliferating K562/4 cells, between 6-25 culture generations, the mean proliferation rate (± SD) determined at 48 hrs was 2.2 ± 0.2 doublings (n=29) from which a mean population doubling time (± S.D) of 22 ± 2 hr (n=29) was estimated.

To measure the effects of drugs on proliferation rates of K562/4 cells, duplicate cultures (20 or 25 ml) were initiated at a population density of 1×10^5 cells/ml in growth media with or without graded concentrations of the drug. The cultures were incubated at 37°C and cells were enumerated at 24-hr intervals for 3 days. Proliferation rates were determined as described above. The % control proliferation rate is the number of population doublings of drug-treated cultures relative to those of untreated (control) cultures.

3) Determination of cell viability

Cell viability was determined by exclusion of trypan blue (Kaltenbach et al 1958). Cells were harvested by centrifugation (100 x g, 8 min) and resuspended (0.5×10^7-$1.0 \text{ X } 10^7$ cells/ml) in sterile phosphate buffered saline (PBS), which consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.4). Equal volumes of cell suspension and trypan blue solution (0.4% (w/v) in 0.15 M NaCl) were mixed and incubated at room temperature for up to 5 min. Viable cells were identified under a light microscope by their ability to exclude dye (100-300 cells were enumerated per assay).

4) Determination of protein content.

Cells (about 5 x 10^5 cells) were collected by centrifugation (100 x g, 8 min), resuspended and washed twice in PBS. In the final centrifugation (15,000 x g, 1 min), the cells were collected in a 1.5-ml microcentrifuge tube and solubilized at room temperature in 1% (w/v) SDS solution. Protein content was determined in the presence of 1% (w/v) SDS by the micro-Lowry assay based on the method described by Markwell et al (1978), using bovine serum albumin as standard.

5) Induction of hemoglobin production

Although treatment of K562 cells with hemin stimulates production of hemoglobin (Rutherford et al 1980), loss of cell viability occurs when cells are treated with high concentrations of hemin. Preliminary experiments were undertaken to find a concentration of hemin that stimulated hemoglobin production without compromising cell viability. Duplicate cultures (1 x 10^5 cells/ml) were exposed to 25, 50 and 100 μM hemin for 4 days and proliferation rates, viability and levels of hemoglobin production were assessed at 24-hr intervals. Hemoglobin production was seen in 70-80% of cells in cultures treated with 50 μM hemin for 3 days and in < 5% of cells in untreated cultures. Viability in hemin-treated cultures ranged between 75-95%.

i) *Induction protocol*

Actively proliferating K562/4 cells (1 x 10^5 cells/ml) were grown at 37°C in the absence (controls) and presence of 50 μM hemin for up to 11 days in RPMI medium supplemented with 50 μM hemin, 5% fetal bovine serum, 5% calf serum and 50 μg/ml gentamicin. The cultures (100 ml) were maintained for 3-4 days without replacing the media. After this period, if the percentage of cells expressing detectable levels of hemoglobin exceeded 70%, cultures to be used for transport or NBMPR-binding assays were transferred to 0.5-L roller bottles, diluted to 2 x 10^5 cells/ml with fresh growth media (with or without hemin) and maintained at population densities between 2 x 10^5 and 6 x 10^5 cells/ml by diluting with fresh media at 2-day intervals. Assessment of proliferation rates, viability and hemoglobin production was performed daily for 3 days and at 2- or 3-day intervals thereafter.

ii) *Identification of hemoglobin-producing cells*

The ability of cells to be stained with 3,3',5,5' tetramethylbenzidine hydrochloride was used to identify hemoglobin-producing cells in hemin-treated cultures. This method, which is a modification of a previously described method (Rowley et al 1981), is based on catalysis by hemoglobin of the rapid oxidation of 3,3',5,5' tetramethylbenzidine (an aromatic diamine) by hydrogen peroxide to a series of quinoid forms that are colored green to blue (Tietz 1972).

Cells were collected by centrifugation (100 x g, 8 min), washed three times with PBS and resuspended in PBS at 1 x 10^7 cells/ml. Two volumes (100 µl) of cell suspension were mixed with one volume (50 µl) of benzidine peroxide solution (0.5% 3,3',5,5' tetramethylbenzidine, 0.5% acetic acid, 1.2% H_2O_2) and the mixtures were incubated at room temperature for 10-15 min. The percentage of cells containing dark blue-green deposits (benzidine-positive cells) were identified under a light microscope (150-550 cells were counted per assay).

iii) *Quantitation of hemoglobin (benzidine assay)*

The hemoglobin content of hemin-treated and untreated cells was determined by a modification of a previously described method (Clarke et al 1982). Cells (about 3 x 10^6 cells) were collected by centrifugation (100 x g, 8 min), washed three times with PBS to remove exogenous hemin and lysed by resuspension in 150 µl distilled water. The lysates were stored at -20°C. For analysis, 50 µl of freshly thawed cell lysate were mixed with 100 µl of 0.5 % benzidine solution (0.5% 3,3',5,5' tetramethylbenzidine in 50% acetic acid) and 100 µl of 1% H_2O_2 solution. The mixtures were incubated at room temperature for 25 min in the dark and then diluted with 750 µl of 10% acetic acid. The amount of hemoglobin present in samples was determined by relating the sample absorbance to a standard calculation curve prepared with 5-300 µg/ml hemoglobin read at 660 nm. The protein content of samples was determined by the micro-Lowry assay (Markwell et al 1978).

v) *Cell cycle analysis*

The relative DNA content of control and hemin-treated K562/4 cells was determined by flow cytometry using propidium iodide, a fluorescent DNA-binding agent (Clevenger et al 1985). Two procedures were used for fixing and staining the cells.

i) Fixation with paraformaldehyde. Cells (1×10^6 - 2×10^6 cells) were collected by centrifugation (100 x g, 8 min), resuspended in 1 ml cold paraformaldehyde solution (0.5% w/v in PBS) and incubated at 4°C for 3 min. The fixed cells were centrifuged (100 x g, 8 min) and permeabilized by resuspension in 1 ml of 0.1% Triton X-100 (4°C, 3 min). The permeabilized cells were collected by centrifugation (100 x g, 8 min), treated with RNase (1 mg/ml in PBS, 20 min, 37°C), collected by centrifugation (100 x g, 8 min), resuspended in 1 ml propidium iodide staining solution (50 μg/ml propidium iodide in 0.1% sodium citrate buffer), and incubated at 4°C for at least 1 hr. Samples, which were kept at 4°C in the dark, were filtered through a 48-μm filter before analysis by flow cytometry.

ii) Fixation with ethanol. Cells (1×10^6 - 2×10^6 cells) were collected by centrifugation (100 x g, 8 min), resuspended in 2 ml 50% fetal bovine serum/50% RPMI medium, resuspended (with vortexing) in 6 ml of cold 70% ethanol and incubated at 4°C for 30 min. The fixed cells were collected by centrifugation (100 x g, 8 min), resuspended in staining solution (10 μg/ml propidium iodide and 100 μg/ml RNase in 0.150 M NaCl) and incubated in the dark for 20 min at room temperature. Cells were collected by centrifugation (100 x g, 8 min), washed once and resuspended (1 ml) in PBS.

Ethanol-fixed cells could be stored at 4°C for several days without change in DNA distributions whereas the paraformaldehyde-fixed cells were stained and analyzed immediately after fixation since the DNA distributions changed with storage.

Fluorescence intensity, a measure of relative DNA content of propidium iodide-stained cells, was determined with a FACScan fluorescence activated cell analyser (Becton Dickinson, Mississauga, Ont.) equipped with an argon laser (peak emission 488 nm) used at an output of 15 mW. Fluorescence was detected after filtering with a 585/42 nm bandpass filter. DNA distributions were analysed with a DNA Cell-Cycle Analysis Software-Ver C program according to a polynomial model.

C. TRANSPORT AND BINDING MEASUREMENTS WITH INTACT CELLS

1) Composition of solutions used for assays

The media in which cells were suspended for transport assays are listed below and differed depending on the nature of the assay. For each one, the pH was adjusted to 7.4, and the osmolarity was measured by freezing-point depression with a µOsmomette Microosmometer (Model 5004; Precision Systems INC., Circle Natick, MA) to ensure that the solutions were isotonic.

i) *Transport RPMI (TRPMI) medium*

TRPMI medium consisted of $NaHCO_3$-free RPMI 1640 medium (GIBCO) that was buffered with 20 mM Hepes (pH 7.4) and supplemented with 5 mM glucose. The osmolarity of the medium was adjusted to 300 ± 15 mOsm by the addition of 1.0 mM NaCl.

ii) *Sodium medium*

Sodium medium consisted of Dulbecco's salts medium (Dulbeco & Vogt 1954) modified by the addition of glucose and contained 1.0 mM $CaCl_2$, 2.6 mM KCl, 1.4 mM KH_2PO_4, 8.0 mM Na_2HPO_4, 138 mM NaCl and 5.0 mM glucose.

iii) *Sodium-free media*

Media that lacked sodium were formulated by substituting either KCl or N-methyl glucammonium chloride for NaCl in sodium medium and consisted of 138 mM KCl or 138 mM N-methyl glucamonium chloride, 1.0 mM $CaCl_2$, 1.4 mM KH_2PO_4, 8.0 mM K_2HPO_4, and 5.0 mM glucose.

iv) *Transport oil*

The oil mixture used for transport assays was prepared by mixing 75 ml paraffin oil (Fisher Scientific, Ottawa Ont.) with 425 ml silicone 550 oil (Dow Corning, Mississauga, Ont) to give a final specific gravity of 1.020-1.033 g/ml. Specific gravity was determined using a Specific Gravity/Baume Hydrometer.

2) Procedures for measurement of transport

Rates of transport of nucleosides were determined under zero-*trans*[1] conditions by modifications of previously described methods

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[1] Under zero-*trans* conditions, the rate of transport of substrate (^3H-permeant) from one face of the membrane (the *cis* face) to the other (the *trans* face) is measured when the concentration of substrate at the *trans* face is zero (Stein 1986).

(Harley et al 1982). Initial rates of uptake of ^{3}H-nucleosides by suspended cells were determined using rapid sampling procedures in which the transport process was terminated either by (i) separating the cells from the permeant-containing solution by centrifugation through a layer of oil with a specific gravity between those of cells and water (Oil stop method), or (ii) quenching by addition of a solution containing transport inhibitor, followed by centrifugation through oil (Inhibitor-oil stop method). Dilazep, a potent inhibitor of equilibrative transport in erythrocytes and several cell lines (Paterson et al 1984) was used as a stopping reagent in the inhibitor-oil stop method.

i) *Transport assay*

Cells were harvested from actively proliferating cultures by centrifugation (100 x g, 8 min) and resuspended at 1 x 10^7 to 2 x 10^7 cells/ml in TRPMI medium. Cell suspensions were used within 20-50 min of harvesting and, where necessary, multiple harvests were prepared in succession from replicate cultures. Cell counting was performed for each harvest of cells as follows. Portions (100 µl, 4 samples) of cell suspensions were dispensed with an Eppendorf Repeater Pippeter into 19 ml physiologic saline solution (150 mM NaCl) and cell numbers were determined by electronic particle counting.

Unless otherwise indicated, uptake assays were performed at 37°C (in a constant temperature room). Transport assays were performed in duplicate or triplicate and each experiment was repeated

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2-4 times. The assays were conducted in 1.5-ml polypropylene microcentrifuge tubes that contained 100-µl portions of ^{3}H-labelled (4-40 µCi/ml) nucleosides in TRPMI medium layered over 100 µl of transport oil. The assay tubes were placed in the rotor of an Eppendorf microfuge (Model 5412 or 5414) opposite a counterweight tube.

For uptake intervals $\leq$ 6 sec, each sample was processed individually and permeant fluxes were stopped using the inhibitor-oil stop method. To begin the uptake interval, a 100-µl portion of the cell suspension was added to the ^{3}H-nucleoside-containing solution in the waiting microfuge tube with an Eppendorf Repeater Pippeter. To end the uptake interval, defined by metronome signals, a 200-µl portion of ice-cold TRPMI medium containing 200 µM dilazep dihydrochloride was added and the tube was immediately centrifuged (15,600 x g, 30s), thereby pelleting the cells under the oil.

For uptake intervals > 6 sec, the assay tubes were placed in the rotor head in groups of 6. Intervals of uptake were begun by adding 100-µl portions of cell suspension to the waiting microfuge tubes at timed intervals, defined by metronome signals, and were ended simultaneously by the oil-stop method. The time interval required for removal of cells from the aqueous medium to form a pellet under the oil has been estimated to be 2 sec (Paterson et al 1984) and, since this time is not negligible with respect to rates of nucleoside transport, uptake intervals were considered to be 2 sec longer than the time elapsed between permeant addition and centrifuge switch-on.

The ^{3}H-labelled nucleoside associated with cell pellets at time "0" was determined by adding the dilazep solution to the permeant solution before adding the cells. Addition of cells was followed by immediate centrifugation.

ii) *Determination of intracellular volume*

Intracellular water volume was estimated by subtracting the extracellular pellet volume (determined from the ^{14}C-sucrose content of the cell pellet) from total pellet water volume (determined from the $^{3}H_2O$ content of the cell pellet). Microfuge tubes containing 100-µl portions of $^{3}H_2O$ or ^{14}C-sucrose in TRPMI medium layered over 100 µl of oil were placed in the microfuge rotor in groups of 4 or 6 tubes (cell volume was determined at least in quadriplicate) and 100-µl portions of cell suspension were added to each of the tubes. After 30-45 sec, the cells were separated from the ^{3}H- or ^{14}C-containing media by centrifugation (15,600 x g, 30 sec) through the oil. These measurements, which were conducted for each transport experiment, were highly consistent between experiments. The mean (± SD) intracellular water volume constituted 90 ± 3% (n = 13) of the pellet volume and was $2.0 \pm 0.3 \times 10^{-6}$ µl/cell (n = 14).

iii) *Sample processing*

The ^{3}H- or ^{14}C-containing solution in the assay tubes was removed by aspiration. The tube walls above the oil layers were washed twice with distilled water, which was removed by aspiration. In the last washing step, the oil layer was also removed and the cell pellets were solubilized in 250 µl of 5% Triton X-100, with gentle mixing (Eppendorf rotatory shaker, 15-30 min). The tubes were then placed in scintillation vials together with 8 ml Triton X-100 based scintillant for determination of radioactivity by liquid scintillation counting (Pande 1976).

4) Determination of concentration-effect relationships for inhibition of transport by NBMPR

The effect of NBMPR on uridine, adenosine and thymidine zero-*trans* influx was determined by a procedure described earlier (Hogue et al 1990) that minimized depletion of NBMPR from the incubation medium. K562/4 cells, which had been harvested from actively proliferating cultures, were incubated at 1 x 10^6 cells/ ml in TRPMI medium containing graded NBMPR concentrations (0.1 nM - 35.0 µM) for 15-20 min at 37^{o}C. These conditions were shown in binding experiments to allow equilibration between bound and free NBMPR without significantly changing the concentration of free NBMPR. The cell suspensions were then centrifuged (100 x g, 8 min) and individual samples were resuspended (1 x 10^7 to 2 x 10^7 cells/ml) in fresh TRPMI medium containing NBMPR at the same concentrations used during the initial incubations. Transport assays were then performed as described above using permeant solutions that also contained NBMPR at the same concentrations as in the preceeding steps.

5) Calculation of transport rates

For each uptake determination, the pellet content of ^{3}H-labelled nucleoside (cpm/pellet) was corrected for radioactivity present in the extracellular space by dividing the total pellet radioactivity (cpm/pellet) by the intracellular pellet volume (µl/pellet). Thus, each uptake value was expressed as an intracellular concentration (pmol nucleoside equivalents/µl of cell water).

In this work, transport was defined as the initial rate of cellular uptake of ^{3}H-nucleoside. Transport rates were derived by analysis of time courses of uptake by (i) linear regression, when time courses were linear, and (ii) second order polynomial regression as the coefficients of the first order term, when time courses were non-linear. Transport rates, which were calculated as pmol nucleoside equivalents/µl cell water/sec, were expressed in a compact form as µM/sec.

f) Analysis of transport kinetics

Under zero-*trans* (zt) conditions, the rate of transfer of permeant molecules from the *cis* to the *trans* face of a membrane reaches a limiting maximum velocity as the permeant concentration is increased (Stein 1986). This limiting velocity has been denoted V_{12}^{zt}, where the *cis* and *trans* faces of the membrane are designated 1 and 2, respectively. The substrate concentration at the *cis* face at which one-half of this maximum velocity is reached has been denoted as K_{12}^{zt}. The hyperbola, $v_{12}=\frac{S_1V_{12}^{zt}}{(K_{12}^{zt}+S_1)}$ (Equation 1), describes the dependence of the rate of transport, v_{12}, from the *cis* to the *trans* face on the substrate concentration, S_1, at the *cis* face of the membrane. The limit of the ratio v_{12}/S_1, as S_1 approaches zero, defines the limiting permeability, $\pi_{12}^{zt}=\frac{V_{12}^{zt}}{K_{12}^{zt}}$ (Equation 2).

Equation (1) has the form of the Michaelis-Menten equation,

$v = \frac{S V_{max}}{(K_m + S)}$ (Equation 3). Transport data are usually analyzed by the same procedure as for enzymes. The limiting permeability, π^{zt}_{12}, is analogous to the first order rate constant, $k = \frac{V_{max}}{K_m}$, of enzyme kinetics. The physical significance of π^{zt}_{12} is that it approximates the fraction of the permeant present at the *cis* face of the membrane that is transported to the intracellular face per unit of time.

In this study, kinetic constants were calculated from data relating transport rates (v_{12}) and nucleoside concentrations (S_1) according to Equation 1. The methods used to calculate kinetic constants were: (a) least squares analysis of plots of S_1/v_{12} versus S_1 and v_{12} versus v_{12}/S_1 and (b) direct linear plots, described by transformations of Equation 1 to linear forms analogous to transformations of the Michaelis Menten equation (Stein 1986, Segel 1976a). The goodness of fit of linear replots of S/v versus S and v versus v/S was assessed by computation of the correlation coefficient (R^2). For convenience, the transport parameters K^{zt}_{12} and V^{zt}_{12} are referred as K_m and V_{max}, respectively.

The direct linear plot is less sensitive to errors of individual observations than least squares estimates and thus does not require weighting of the data. The equation describing the direct linear plot is $V_{max} = v + \frac{v}{S} K_m$. V_{max} and K_m are treated as variables and v and S as constants. If S values are plotted on the negative horizontal axis and the corresponding v values are plotted on the vertical axis, straight lines connecting each pair of -S and v values intersect at points with the coordinates: $S = K_m$ and $v = V_{max}$. Each line plotted for a pair (v, S) relates all possible pairs of K_m and V_{max} values that satisfy an observation of rate v corresponding to a substrate concentration S. If there is no experimental error, n lines for a set of n observations would intersect at a unique point, the coordinates of which would give the values of Km and Vmax for the system under study. However, experimental values yield a group of intersection points, thus providing a number of estimates of Km and Vmax values, the best of which is the median value of the set.

6) Equilibrium binding of ^{3}H-NBMPR by intact cells

Binding of NBMPR to K562 cells was measured by a modification of a previously described procedure (Cass et al 1981). Cells were harvested from actively proliferating cultures, washed once and resuspended in TRPMI medium as in transport assays. Binding assays were initiated by adding 500-µl portions of cell suspensions (10^6 cells/ml) to 500-µl portions of TRPMI medium that was layered over 100 µl transport oil in microfuge tubes. The TRPMI media contained graded concentrations (0.1-5.0 nM) of ^{3}H-NBMPR alone or with an excess of (10 µM) non-radioactive NBMPR or nitrobenzylthioguanosine (NBTGR) for determination of nonspecific binding. To achieve equilibrium between bound and free ligand, the mixtures were incubated for 30 min at room temperature or, where noted, 37°C. Binding assays were terminated by centrifugal pelleting (15,600 x g, 1 min) of cells under the oil layer. Concentrations of free ^{3}H-NBMPR were determined from the radioactivity present in cell-free media after centrifugation and the amounts of bound ^{3}H-NBMPR from the pellet-associated radioactivity. Processing of samples for determination of radioactivity was similar to that described for transport experiments.

Site-specific binding of NBMPR was calculated as the difference between total pellet-associated radioactivity and pellet-associated radioactivity determined in the presence of 10 μM non-radioactive NBMPR or NBTGR. Values for maximum binding activity, Bmax, and the dissociation constant, Kd, were estimated by mass law analysis of equilibrium binding data, according to the method of Scatchard (Segel 1976 b). The concentration range employed to study high-affinity binding of ^{3}H-NBMPR in K562/4 cells was determined in preliminary experiments in which saturability of ^{3}H-NBMPR binding was examined within a range of 0.1-100 nM.

D. STUDY OF MEMBRANE POLYPEPTIDES PHOTOLABELLED WITH ^{3}H-NBMPR

1) Preparation of membrane fractions

i) *K562/4 cells*

Membrane fractions enriched in plasma membranes were prepared with minor modifications by a previously described method (Hogue et al 1990). Cells were harvested by centrifugation (150 x g, 10 min), washed twice with ice-cold PBS and resuspended (about 20 x 10^6 cells/ml) in ice-cold hypotonic Tris buffer (10 mM Tris-HCl, 10 mM NaCl, 1.5 mM $MgCl_2$, 1.5 mM dithiothreitol, 0.1 mM PMSF, 1.0 mM EDTA-Na_2, pH 6.8 at 25° C). All subsequent procedures were conducted at 4°C. After being incubated for 15 min in hypotonic Tris buffer, the swollen cells were disrupted by sonication (Brausonic 1510 sonicator) and centrifuged (2500 x g, 15 min). Membrane pellets were collected from the slow-speed supernatants by further centrifugation (50,000 x g, 30 min), suspended in 20% Percoll sucrose solution (20% v/v Percoll, 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA-Na_2, pH 7.4 at 4°C) and subjected to Dounce homogenization (type A, 20-30 strokes). The homogenates were centrifuged (35,000 x g, 30 min) and membrane fractions were separated in a 10-ml self-generated Percoll gradient. Fractions enriched in plasma membranes (present as a thin layer at the top of the gradient) were isolated and stored in liquid nitrogen. Percoll was removed before further use of the membrane preparations by two washes (89,000 x g, 20 min, followed by 150,500 x g, 20 min, in a Beckman TL100.3 rotor) in 50 mM Tris buffer (50 mM Tris-HCl, pH 7.4 at 4°C). The membranes were recovered as a tight layer above the sedimented Percoll.

Density marker beads (Pharmacia Dorval, Que.) were used to identify conditions (% of Percoll, speed and time of centrifugation) that produced gradients in the low density range (between 1.037-1.069 g/ml) for separation of the light plasma membrane-enriched fraction from the heavier mitochondrial fraction.

ii) *Human erythrocytes*

Outdated erythrocytes were obtained from the Red Cross Blood Transfusion Service (Edmonton, Alberta). About 150 ml of packed cells were washed 3 times by centrifugation (400 x g, 15 min, 4°C) and resuspension in ice-cold PBS. Hemoglobin-free membranes were prepared by hypotonic lysis by a modification of the procedure described by Dodge et al. (1963) and, unless otherwise stated, all manipulations were at 4°C.

One volume of washed erythrocytes was mixed with 10 volumes of ice-cold hypotonic phosphate buffer (5 mM Na_2HPO_4, 0.2 mM PMSF, 0.1 mM EDTA, pH 8.0) and gently stirred for 15 min to lyse the swollen cells. The membranes were collected by centrifugation (16,080 x g, 30 min, 4°C) and washed repeatedly until white to light pink in color with hypotonic phosphate buffer to remove free hemoglobin. The membranes were further depleted of extrinsic proteins as described by Jarvis et al. (1981). Briefly, hemoglobin-free membranes were stirred for 25 min with 4 volumes of ice-cold 0.1 mM EDTA (pH 11.2) and the resulting protein-depleted membranes were collected by centrifugation (17,570 x g, 60 min) and washed twice by centrifugation (10,310 x g, 20 min) and resuspension in Tris buffer. The membrane suspensions were either used immediately or stored in liquid nitrogen. Protein content was determined in the presence of 1% (w/v) SDS by the micro-Lowry assay (Markwel et al 1978).

2) Binding of ^{3}H-NBMPR to membranes

Binding of NBMPR to membranes was assessed by centrifugal gel filtration using a modification of a previously described method (Jarvis & Young 1987). All manipulations were at 4°C, unless otherwise stated. Sephadex G-50 (coarse) was equilibrated overnight in Tris buffer and, after degassing (1-2 hrs at 22°C), was packed in disposable 1-ml syringes (0.5 x 16 mm) and allowed to drain. Just before use, the columns were centrifuged (1000 x g, 2 min). Plasma-membrane enriched fractions from K562 cells or protein-depleted membranes from erythrocytes (about 0.4 mg of protein/200 µl assay mixture) were incubated (75 min, 4°C) in Tris buffer containing 50 nM ^{3}H-NBMPR in the presence or absence of 7.5-10 µM non-radioactive NBMPR. An 80-µl portion of the mixture was added to the column, which was suspended in a mini scintillation vial, and, after about 15 sec, 20 µl of Tris buffer was added and the column was centrifuged (1000 x g, 2 min). The eluent, which contained ^{3}H-NBMPR bound to membrane-suspension fractions, was assayed directly for ^{3}H-content by liquid scintillation counting in a Triton X-100 based scintillant (Pande 1976). Specific binding (cpm/µg protein) was estimated by subtracting the ^{3}H-content of the sample incubated with ^{3}H-NBMPR in the presence of 10 µM NBMPR (non-specifically bound) from the ^{3}H-content of the sample incubated in the absence of NBMPR.

3) Photoaffinity labelling of membranes with ^{3}H-NBMPR

Plasma-membrane enriched fractions from K562 cells or protein-depleted membranes from erythrocytes were photolabelled with ^{3}H-NBMPR under conditions that allowed equilibration of binding (Gati et al 1986). Portions of the membrane-containing preparations (1-3 mg membrane protein/ml reaction mixture) were incubated for 75 min at 4°C in Tris buffer containing 50 nM ^{3}H-NBMPR alone or together with 7.5-10 µM non-labeled NBMPR for determination of non-specific binding. The mixtures, which also contained 10 mM dithiothreitol to scavenge free radicals generated by uv irradiation (Young et al 1983), were transferred to 60 x 15-mm plastic petri dishes resting on ice. The dishes (with the lids on) were irradiated for

3 min with uv light at a distance of 4 cm from the quartz cooling sleeve of a 450-W mercury-arc lamp (Canrad-Hanovia Inc. Newark, NJ, USA). The suspensions were then diluted 5-fold with Tris buffer containing 10 μM non-labelled NBMPR and allowed to stand at room temperature for about 15 min (for displacement of non-covalently bound ^{3}H-NBMPR by non-labelled NBMPR). The membrane fractions were recovered by centrifugation (50,000 x g, 30 min, room temperature) and washed once with Tris buffer containing 10 μM non-labeled NBMPR. Finally the photolabelled membranes were resuspended in Tris buffer and small portions (10-20 μl) were withdrawn for determination of protein and ^{3}H-content. One-ml portions of the photolabelled membranes were stored in liquid nitrogen.

4) SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out in 1.5- to 3-mm thick slab gels or 1-mm thick mini gels by the method of Thompson & Maddy (1982) using the discontinuous buffer system of Laemmli (1970). Photolabelled membrane fractions were solubilized in 1 to 2 volumes of electrophoresis sample buffer (4% (w/v) SDS, 20% (w/v) glycerol, 0.13 mM Tris at pH 6.8, 0.01% bromophenol blue, 2% mercaptoethanol) and applied to gels without heating (to avoid aggregation of NBMPR-labelled polypeptides (Wu et al 1984). Molecular weight markers were applied in adjacent lanes and their migration distances were determined by staining with Coomassie blue. For determination of migration distances of photolabelled polypeptides, 2-mm gel slices were dissolved in Eqonofluor that contained 3-6 % (v/v) Protosol (New England Nuclear). Samples (in scintillation vials) were incubated at 37°C for 48 h and, after being cooled to room temperature, the ^{3}H-content was determined by liquid scintillation counting. Recovery of radioactivity from the gels was 70-85 %. The Eqonofluor-Protosol scintillation fluid gives low and reproducible background counts (<50 cpm), allowing the analysis of samples with low radioactive content (Jarvis & Young 1987).

5) Deglycosylation of membranes with N-glycosidase F

Enzymatic deglycosylation of photolabelled membranes was conducted according to the protocol recommended by the manufacturers (Genzyme Corp., Boston, MA) with minor modifications. Membranes (about 100 µg protein) were solubilized in about 40 µl of 0.5% (w/v) SDS and 0.1 M mercaptoethanol. The solubilized membranes were then diluted with enzyme reaction buffer (0.2 M Na_2HPO_4, pH 8.6, 0.12% SDS, 0.9 % Nonidet P-40, 10 mM 1.0 phenanthroline hydrate, 0.1 M β-mercaptoethanol, 0.12 mg/ml leupeptin, 1 mM Na_2-EDTA, 1 mM phenylmethyl-sulfonyl fluoride) to a final volume of about 100 µl. N-Glycosidase F (0.120 - 0.190 U/20 µg membrane protein) was added and the reaction mixtures were incubated for 1-2 h at 37°C. Samples were either immediately analysed by SDS-polyacrylamide gel electrophoresis or frozen at -20°C to be analysed later. Photolabelled membranes that were subjected to the same manipulations in the absence of enzyme served as controls. The migration distances of radiolabelled polypeptides were determined by analysis of the ^{3}H-content of 2-mm gel slices and of molecular weight markers by staining with Coomassie blue.

6) Proteolysis of membranes with V8 protease

Photolabelled membranes were analyzed by an adaptation of Cleveland's method (Cleveland et al 1976) for peptide mapping in which limited proteolysis with *Staphylococcus aureus* V8 protease took place while photolabelled polypeptides and enzyme migrated together in the stacking gel during SDS-polyacrylamide gel electrophoresis. Membranes (100-400 µg/sample) were solubilized in electrophoresis sample buffer and placed in the wells of 4% stacking gels (2.5 or 5 cm in length) alone or together with V8 protease. Electrophoresis was initiated at currents of 35-40 mA. Proteolytic digestion was carried out as membrane proteins and enzyme migrated along the stacking gel. To vary the length of digestion the current was turned-off for 15-30 min when the tracking dye neared the bottom of the stacking gel. The resulting peptide fragments were separated in a 12-15 % separating gel (10 cm in length) at currents of 50-70 mA and the migration distances of photolabelled fragments were determined by analysis of the ^{3}H-content of 2-mm gel slices as described above.

CHAPTER III

CHARACTERIZATION OF THE NUCLEOSIDE TRANSPORT PROCESS IN K562/4 CELLS

A. OVERVIEW

The studies described in this chapter were undertaken to describe mediated nucleoside permeation processes in cultured K562 human erythroleukemia cells. Experiments were performed with a previously characterized subclone, K562/4, which was derived from cells obtained from the ATCC (Sutherland 1984). The ATCC line, which has been maintained in continuous culture for several years, exhibits considerable cellular heterogeneity in expression of various markers of erythroid differentiation (Lozio & Lozio 1979, Anderson et al 1979) and it seemed likely that cells of the ATCC line might also exhibit heterogeneity in nucleoside permeation characteristics.

Permeation of the physiologic nucleosides, adenosine and uridine, was examined in detail since both are known to be substrates for the equilibrative and concentrative transport systems described in other cell types (Gati & Paterson 1989). As well, adenosine and uridine transport have been studied extensively in human erythrocytes (Cabantchic & Ginburg 1977, Plagemann et al 1985). Since transport processes are, in general, highly temperature dependent (Stein 1986, Plageman & Wohlhueter 1984, Jarvis & Martin 1985), the studies reported here were conducted at physiological temperature (37°C) rather than at room temperature. Most published studies of nucleoside transport kinetics have been conducted at room temperatures because of difficulties in accurately determining initial rates at 37°C.

The existence of nucleoside transport processes can be recognized from the kinetic properties of nucleoside fluxes. In cells that metabolize nucleosides, fluxes are measured in the inward direction and the resulting time courses represent the combined results of (i) influx and backflux, and (ii) metabolic conversion, most commonly phosporylation (Wohlhueter & Plagemann 1980). Current approaches to measure inward fluxes are based on the concept that the initial rate of cellular uptake of isotopic nucleoside describes its rate of inward transport (Harley et al 1982). In this work, rapid sampling technology developed previously (Harley et al 1982, Paterson et al 1981) for studies of nucleoside influx into suspended cells was adapted for determination of initial rates of uptake of ^{3}H-nucleosides by K562/4 cells at 37°C.

An objective of this work was to determine if K562/4 cells express an erythrocyte-like nucleoside transport system. Since the transporter of erythrocytes is NBMPR-sensitive, NBMPR inhibition was evaluated in the initial characterization of the nucleoside transport process of K562/4 cells. NBMPR has been a valuable molecule for exploration of the heterogeneity of the nucleoside transport process since the sensitivity of transport to inhibition by NBMPR differs in different cell types. Because it apparently binds to a single set of high-affinity sites in the plasma membrane of erythrocytes, NBMPR has been used to quantitate the number of NBMPR-sensitive transporters in erythrocytes (Cass et al 1974).

The specific aims of the work described in this chapter were:

(1) to determine if initial rates of nucleoside uptake could be measured at 37°C using rapid-assay procedures developed for transport studies with suspended cells,

(2) to evaluate the sensitivity of nucleoside fluxes to inhibition by NBMPR and, if high sensitivity was found, to characterize interaction of NBMPR with cells,

(3) to determine the kinetic parameters of the overall transport process and, if present, of individual components,

(4) to determine if transport component(s) were equilibrative and/or concentrative, and

(5) to evaluate the relative importance of various transport components, in the cellular uptake of cytotoxic nucleosides.

B. RESULTS

1) *Effectiveness of NBMPR and dilazep as stopping reagents in the assay of uridine and adenosine transport by K562/4 cells*

Rapid sampling methods are required to obtain time courses of nucleoside uptake from which transport rates can be defined. For suspended cells, uptake intervals can be ended by (i) centrifugal pelleting of cells under an oil layer, a process that requires a significant interval of time with respect to the rapidity of the transport process (Harley et al 1982), or (ii) use of inhibitors of nucleoside transport as instantaneous chemical stoppers (Harley et al 1982, Paterson et al 1984) in which case uptake is ended by addition of inhibitor, followed by immediate centrifugal pelleting of cells under oil.

NBMPR has been used as a stopping agent to block permeation of adenosine in cultured S49 and L5178Y mouse lymphoma cells (Paterson et al 1981, Harley et al 1982) since with these cells, virtually instantaneous termination of cellular accumulation of adenosine and its metabolites is achieved with rapid addition of NBMPR (final concentrations, 5-20 µM) to assay mixtures. Dilazep is also a potent inhibitor of adenosine permeation (Pohl & Brock 1974) and, being highly soluble in water, can be used in higher concentrations than NBMPR to end uptake intervals. In experiments where cells were exposed simultaneously to adenosine and high concentrations (333 µM) of dilazep, permeation was blocked almost completely in L5178Y and S49 mouse lymphoma cells and HeLa cells (Paterson et al 1984) . In the same study dilazep was used in a quenched-flow apparatus that allowed measurement of cellular accumulation of adenosine by suspended S49 cells during intervals of 50-500 milliseconds.

Because there is a considerable diversity in nucleoside transport systems, the sensitivity of nucleoside permeation to a particular transport inhibitor must be evaluated for each cell-permeant combination under study before employing that inhibitor as a stopping reagent. Consequently, experiments were undertaken (i) to examine the sensitivity of the nucleoside transport process of K562/4 cells to NBMPR and dilazep, the two inhibitors most widely used as quenching agents in assays of nucleoside transport, and (ii) to determine which inhibitor was most effective in stopping transport reactions at 37°C. The objective was to identify conditions that allowed measurements of nucleoside influx within very short (1-2 sec) intervals.

In the experiments of Figure 1, uptake of uridine by K562/4 cells exposed to a high or a low concentration of uridine was measured in the presence or absence of 10 μM NBMPR. In other cell types 10 μM NBMPR completely inhibits nucleoside transport by the NBMPR-sensitive route (Harley et al 1982, Paterson et al 1987). In K562/4 cells, 10 μM NBMPR did not completely inhibit uridine transport. As is better illustrated in the insets, where the time courses obtained in the presence of NBMPR are plotted with an expanded scale, cellular uptake reached levels that were 50% of extracellular concentrations within 1 min, and the extent of the inhibition by NBMPR was less at the higher uridine concentration. These results, which demonstrated the presence of a route of uridine permeation that is not inhibited by 10 μM NBMPR, indicated that NBMPR could not be used as a stopping reagent in studies of nucleoside influx in K562/4 cells.

The results of Figure 1 also show that uridine was rapidly accumulated by K562/4 cells under the conditions of the transport assay. The extent of this accumulation differed at the low and high concentrations of uridine. After a 1-min exposure to 6 μM uridine, the intracellular ^3H concentration (pmol uridine equivalents/μl cell water) was about 10 times greater than the extracellular concentration, and, after exposure to 291 μM uridine, the intracellular ^3H concentration was about 2 times greater. Uridine is rapidly converted to its phosphorylated derivatives in cultured cells (Wohlhueter & Plagemann 1980), and it is likely that the accumulation of uridine observed in the experiments of Figure 1 was due to metabolic trapping inside cells by phosphorylation.

Uridine transport at 37°C by L1210 cells can be quenched by the combined effects of (i) 100 μM dilazep, and (ii) reduced temperature (Hogue et al 1990). The experiments of Figure 2, which were undertaken to determine if a similar procedure could be used with K526/4 cells, show that simultaneous exposure of cells to uridine and an ice-cold solution containing 100 μM dilazep blocked cellular uptake of uridine. During 1 min in the presence of cold dilazep, uridine uptake remained almost constant, with values similar to those at the earliest time points (see Figure 2 insets, where the time courses obtained in the presence of dilazep are plotted with an expanded scale).

The experiments of Figure 3 were undertaken to determine if the ice-cold dilazep-containing solution was effective after initiation of uptake reactions. Cellular uptake of adenosine at low and high concentrations was allowed to proceed for 4 sec, at which time the cold dilazep solution was added and cells were incubated for additional periods, up to 6 sec. Influx was almost completely blocked for up to 4 sec at both the low and high concentrations of adenosine.

Thus, rapid addition of an ice-cold solution of dilazep inhibited uridine and adenosine transport in K562/4 cells for a period of time longer than the 2 sec required to separate cells from the ^{3}H-permeant solution by centrifugation through oil. This quenching procedure was used, unless otherwise noted, in subsequent experiments. An example of time courses that could be achieved with the inhibitor oil-stop procedure are presented in Figure 4.

Figure 1

Effect of NBMPR on uridine uptake

K562/4 cells (1×10^7 cells/ml) were exposed to ^{3}H-uridine (6 and 291 µM, 4.9 µCi/ml) at 37°C for the indicated time intervals in the presence (open circles) or absence of 10 µM NBMPR (closed circles). Assays of cellular uptake of ^{3}H-uridine were conducted using the oil-stop method described in Chapter II, Section C.2. Periods of uptake were started by addition of the cell suspension to the uridine-containing solution and were ended by centrifugation of cells through the oil. Each 60-sec time course was obtained from a group of 6 assay mixtures to which cells were added sequentially with timing by metronome signals. The time intervals indicated for cell exposure to uridine include the 2-sec period required for centrifugation of cells through the oil layer. Uptake in the absence of NBMPR was assayed immediately after harvesting and resuspending cells in TRPMI solution. Uptake in the presence of NBMPR was assayed after cells had been incubated at 37°C in TRPMI solution containing 20 µM NBMPR for 10-15 min. Plotted values are the means of two or three determinations, and error bars (M.D. or S.D., respectively) are shown where deviations were large enough to extend beyond the symbols. The uptake curves for assays conducted in the presence of NBMPR are reproduced with an expanded scale in the insets.

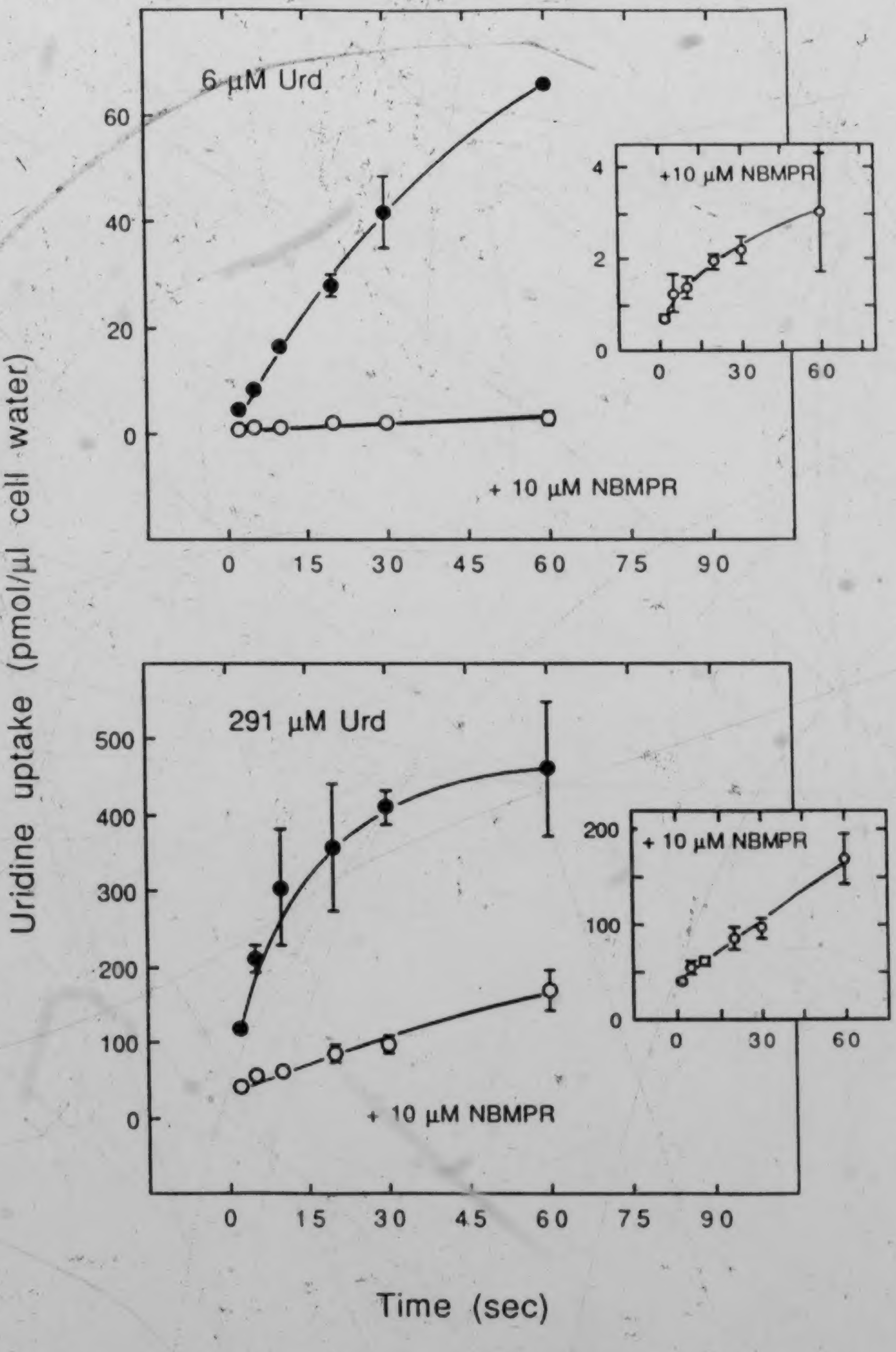

6 µM Urd
+10 µM NBMPR
+ 10 µM NBMPR
291 µM Urd
+ 10 µM NBMPR
+ 10 µM NBMPR
Uridine uptake (pmol/µl cell water)
Time (sec)
0
15
30
45
60
75
90
0
20
40
60
4
2
0
30
60
500
400
300
200
100
200
100

Figure 2

Effectiveness of ice-cold dilazep as a stopping reagent for assay of uridine transport

K562/4 cells (1 x 10^7 cells/ml) were exposed to ^{3}H-uridine (6 and 291 µM, 4.9 µCi/ml) for the indicated intervals in the presence (open circles) or absence (closed circles) of 100 µM dilazep. Each 60-sec time course for cellular uptake of uridine was obtained from a group of 6 assay mixtures using the oil-stop method. Uptake of uridine in the absence of dilazep was performed at 37°C exactly as described in Figure 1. For the assays in the presence of dilazep, 200-µl portions of cold (4°C) TRPMI solution containing 200 µM dilazep were added to 100-µl portions of warm (37°C) ^{3}H-uridine-containing solution, and uptake was initiated at timed intervals thereafter by addition of warm (37°C) 100-µl portions of cell suspension. Plotted values are the means of two or three determinations, and error bars (M.D. or S.D., respectively) are shown where deviations were large enough to extend beyond the symbols. The uptake curves for assays conducted in the presence of dilazep are reproduced with an expanded scale in the insets.

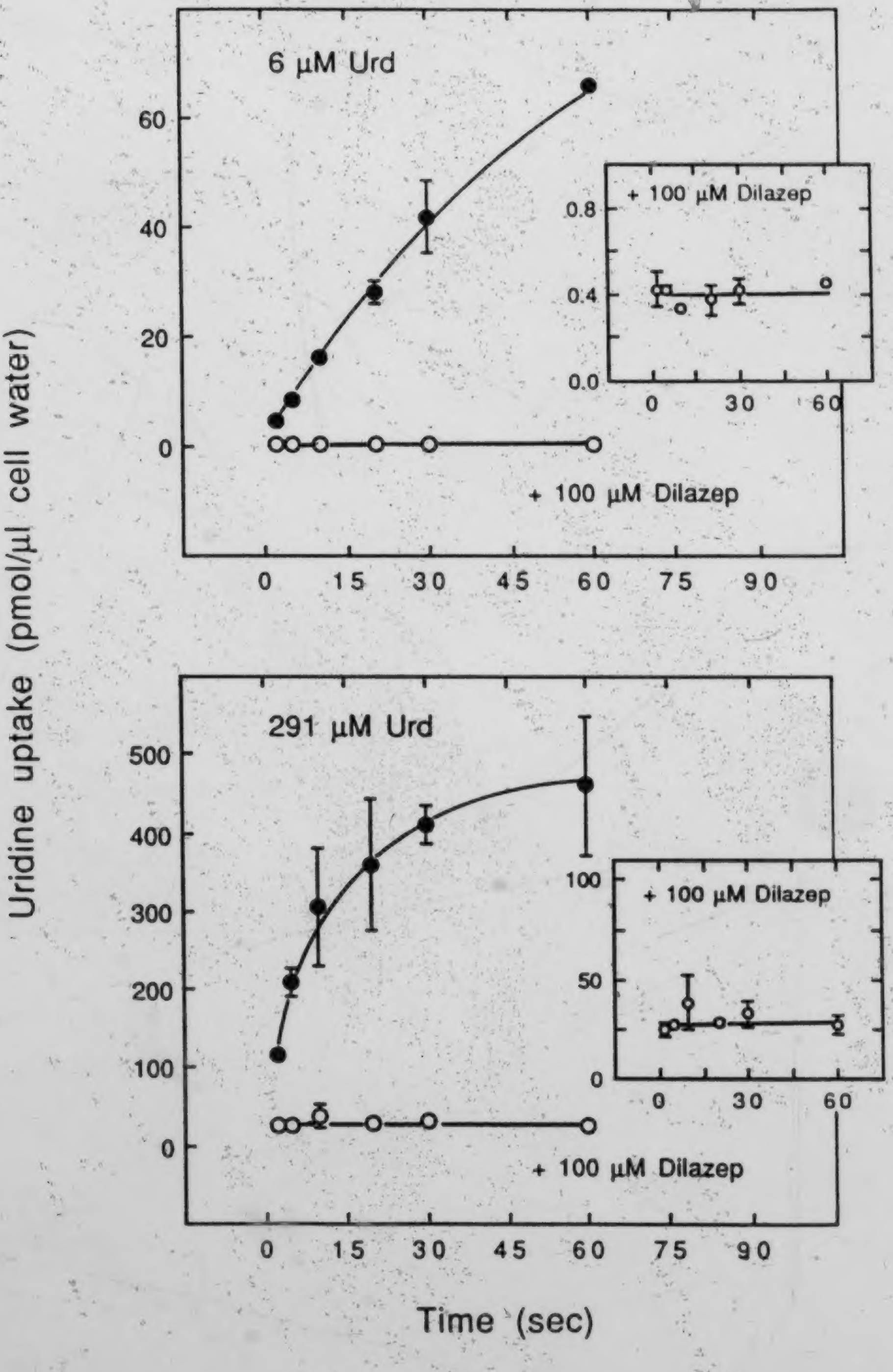

6 µM Urd
60
40
20
0
+ 100 µM Dilazep
0.8
0.4
0.0
0
30
60
+ 100 µM Dilazep
0
15
30
45
60
75
90
Uridine uptake (pmol/µl cell water)
291 µM Urd
500
400
300
200
100
0
+ 100 µM Dilazep
100
50
0
0
30
60
+ 100 µM Dilazep
0
15
30
45
60
75
90
Time (sec)

Figure 3

The use of ice-cold dilazep as a stopping reagent for assay of adenosine transport

K562/4 cells (9 x 10^6 cells/ml) were exposed to ^{3}H-adenosine (1 and 100 μM, 9.4 μCi/ml) at 37°C for the indicated intervals, which were ended by addition of 200 μl of ice-cold transport solution containing 200 μM dilazep. In one set of assay mixtures (open circles), cells were centrifuged through oil immediately after addition of dilazep. In a second set of assay mixtures (closed circles), centrifugation of cells was delayed after addition of dilazep (see arrow) for the periods indicated. Plotted values are the arithmetic means of two determinations, and error bars (M.D.) are shown where deviations were large enough to extend beyond the symbols.

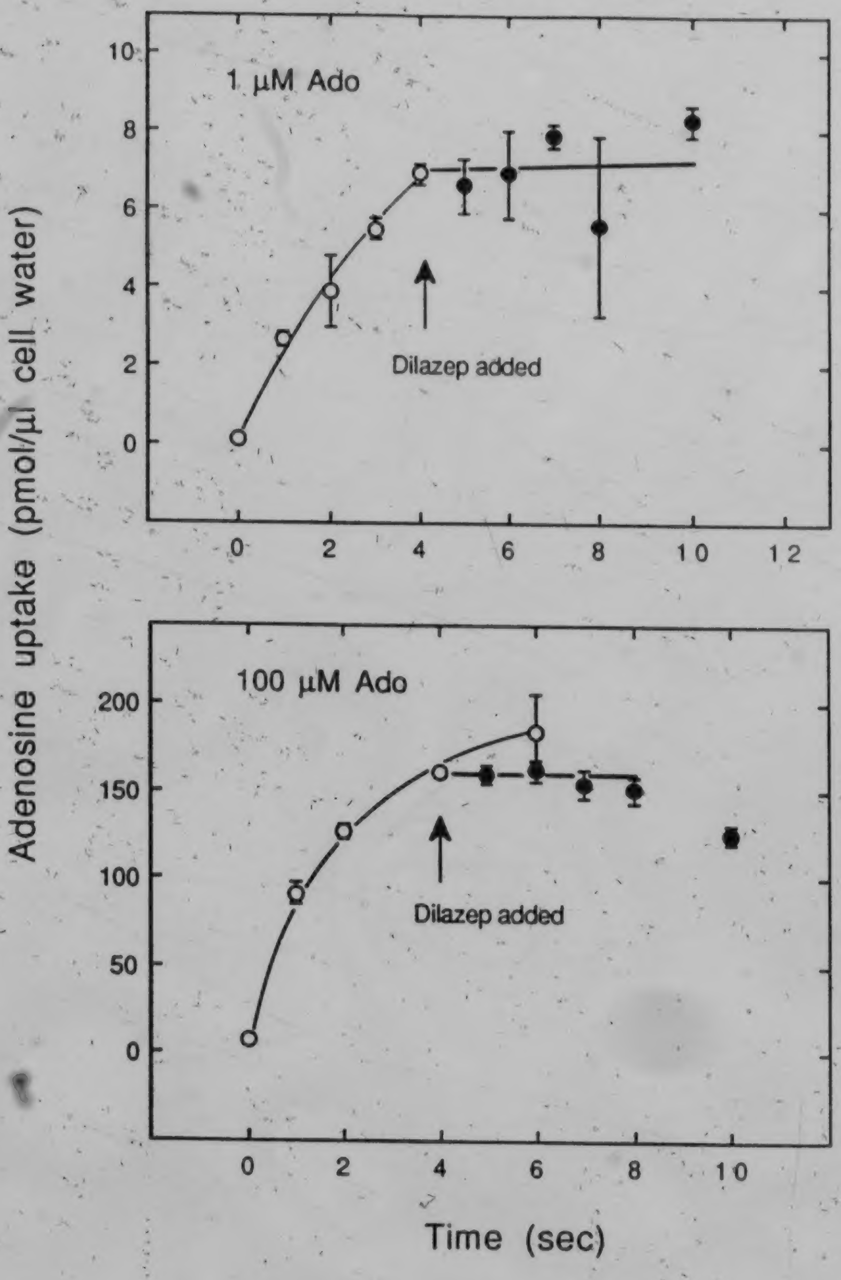
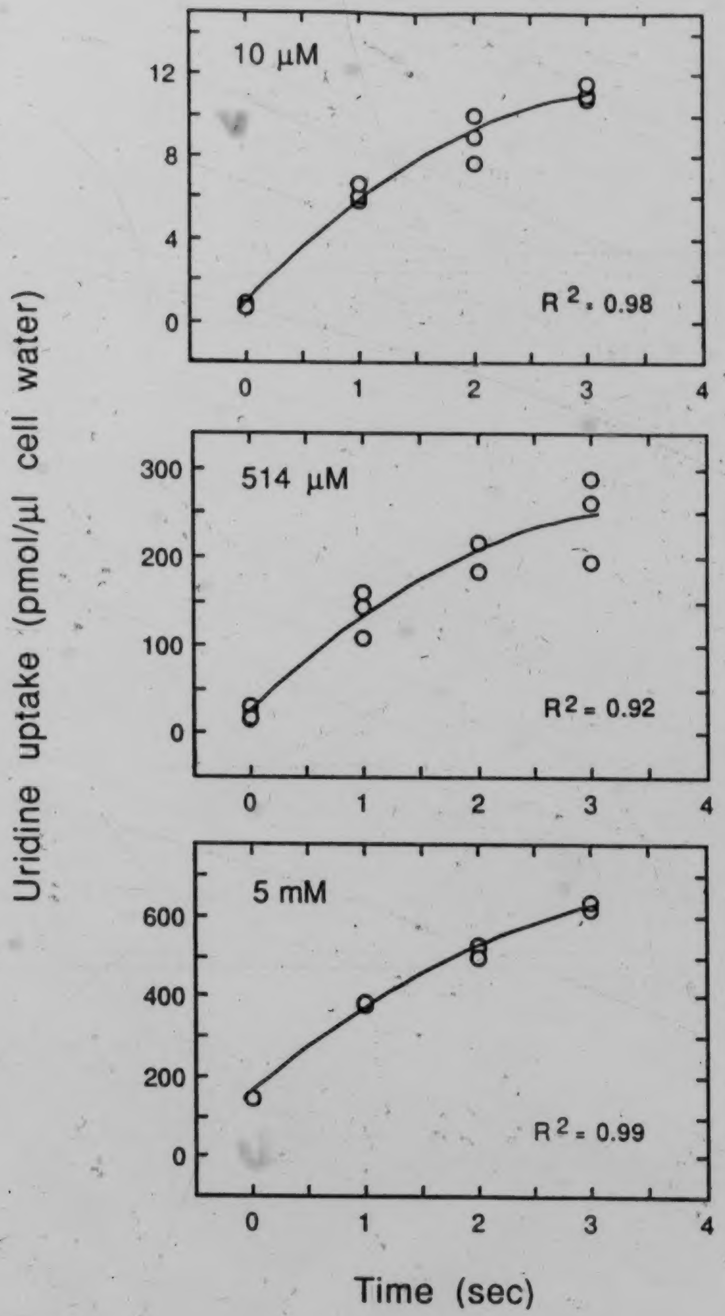

Adenosine uptake (pmol/µl cell water)
1 µM Ado
Dilazep added
10
8
6
4
2
0
0
2
4
6
8
10
12
100 µM Ado
Dilazep added
200
150
100
50
0
0
2
4
6
8
10
Time (sec)

Figure 4

Representative time courses for uridine influx

Influx of ^{3}H-uridine (35 μCi/ml) at 37°C was determined in K562/4 cells (1.0 x 10^5 - 1.5 x 10^5 cells/assay) by the inhibitor-oil stop method. Each point represents a single determination and parabolas were fitted to the data by 2nd order polynomial regression analysis (Macintosh Cricket Graphics program). Transport rates (μM/sec) were: 5.8 (10 μM), 124.4 (514 μM), and 240.6 (5 mM). The correlation coefficients (R^2) of the fitted parabolas are indicated in each graph.


10 µM
12
8
4
0
R 2 = 0.98
0
1
2
3
4
514 µM
300
200
100
0
R 2 = 0.92
0
1
2
3
4
5 mM
600
400
200
0
R 2 = 0.99
0
1
2
3
4
Uridine uptake (pmol/µl cell water)
Time (sec)

2) *Characterization of the inhibition of uridine, adenosine and thymidine influx by NBMPR*

The failure of 10 μM NBMPR to completely inhibit uridine transport in the experiments of Figure 1 suggested that nucleoside permeation in K562/4 cells occurs by both NBMPR-sensitive and NBMPR-insensitive routes. The existance of multiple permeation routes, which differ in sensitivity to inhibition by NBMPR, has been shown in other cell types by determination of concentration-effect relationships for NBMPR inhibition of transport (Paterson et al 1987, Cass et al 1987, Plagemann & Wohlhueter 1984a). IC_{50} values for NBMPR sensitive transport range between 0.1-1.0 nM. Nucleoside permeation by NBMPR-insensitive routes is unaffected by concentrations of NBMPR as high as 1-10 μM.

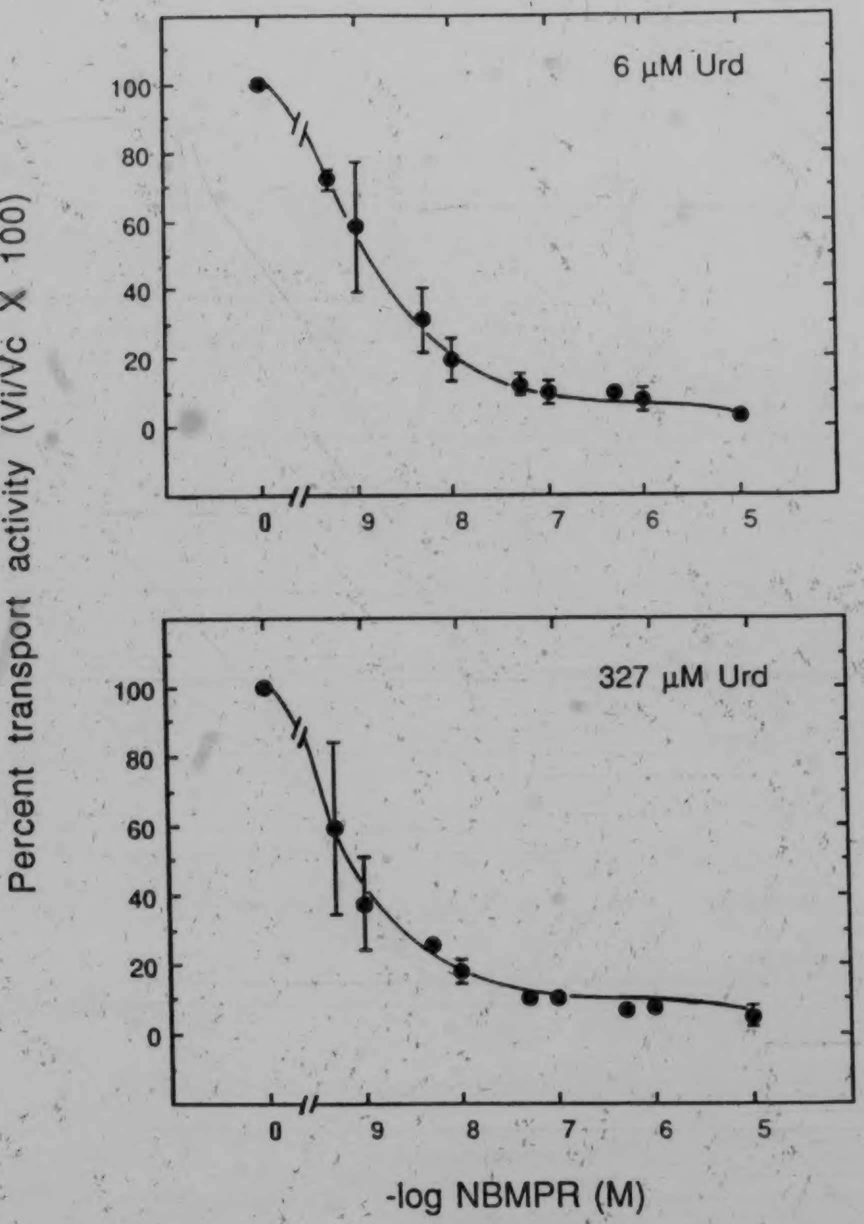
The NBMPR sensitivity of nucleoside permeation in K562/4 cells was examined in the experiments of Figure 5, which measured the effects of a broad range of concentrations (0.1 nM - 35 μM) of NBMPR on zero-*trans* influx of three nucleosides (uridine, thymidine, adenosine). A biphasic concentration-effect relationship was obtained for NBMPR inhibition of influx of all three nucleosides. About 90% of influx activity was blocked by nanomolar concentrations of NBMPR, with IC_{50} values of 0.4-1.8 nM for the NBMPR-sensitive processes. Inhibition reached a broad plateau that spanned the concentration range of 5 nM to 1 μM NBMPR, and micromolar concentrations were required to achieve any further inhibition. Results from several experiments are summarized in Table I.

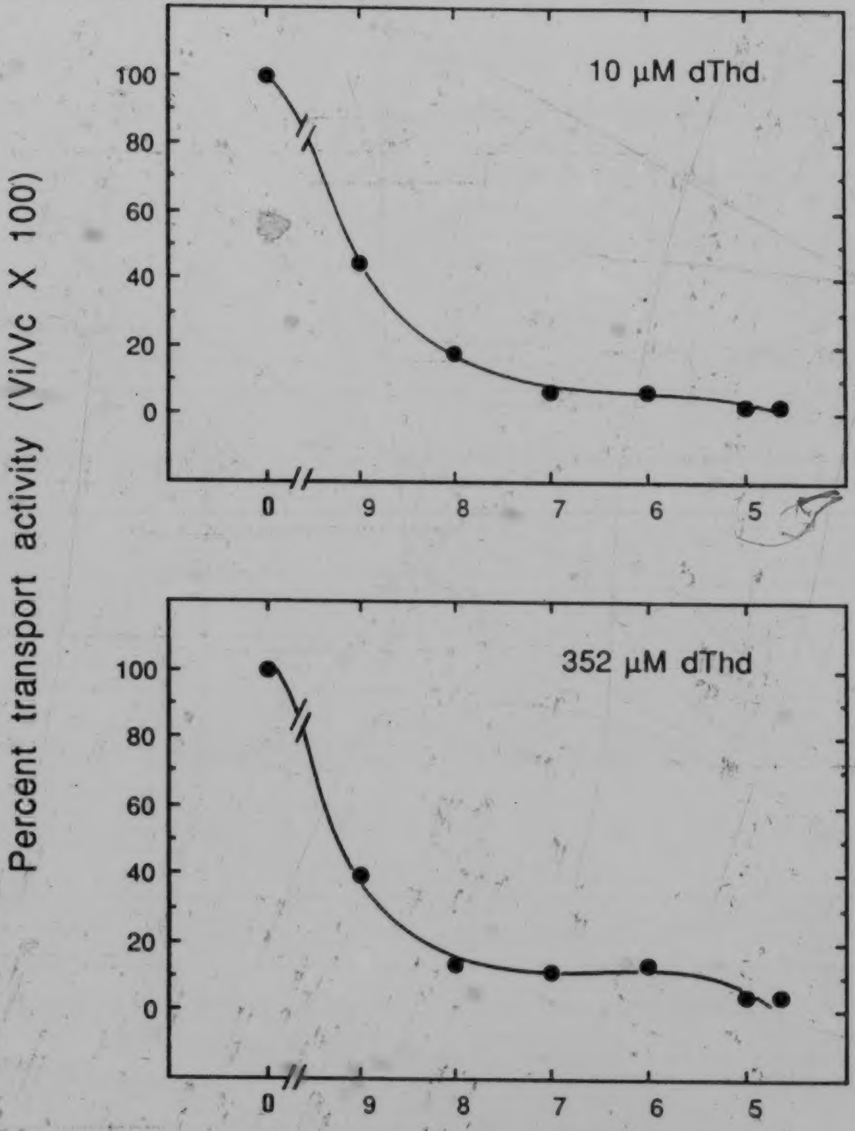
The biphasic nature of the concentration-effect curves for NBMPR inhibition suggested the presence of at least two processes

Figure 5

Concentration-effect relationships for inhibition by NBMPR of uridine, adenosine and thymidine zero-*trans* influx

The effect of NBMPR on cellular uptake of ^{3}H-uridine (6 μM or 327 μM, 20.8 μCi/ml), ^{3}H-adenosine (10 μM, 3.4 μCi/ml) and ^{3}H-thymidine (10 μM or 352 μM, 7.4 μCi/ml) by K562/4 cells was determined as follows: Cells were first incubated (15-20 min, 37°C, 4 x 10^5-1.7 x 10^6 cells/ml) with graded concentrations of NBMPR (0.1 nM-35 μM) and then harvested (0.4 x 10^7-1.7 x 10^7 cells/ml) and assayed at 37°C, for transport in the presence of the same concentrations of NBMPR. Velocities of zero-*trans* influx were calculated from uptake time courses after exposures of 0, 2, 4 and 6 sec (for 6 μM uridine) or 0, 1, 2, and 3 sec (for 327 μM uridine, adenosine and thymidine). Uptake of 2 concentrations was assayed per harvest. Results are expressed as the velocity fraction (Vi/Vc), which represents the ratio of influx estimated in the presence (Vi) or the absence (Vc) of NBMPR (i and c symbolize inhibited or control, respectively). Values for uridine are the means (± S.D. or M.D.) from 3 (6 μM) or 2 (327 μM) experiments and for adenosine and thymidine are from single experiments. Control rates (μM/sec) were: 2.3 ± 0.9 (6 μM uridine), 67.0 ± 25.0 (327 μM uridine), 10.9 (10 μM adenosine) 6.3 (10 μM thymidine) and 69.6 (352 μM thymidine).


Percent transport activity (Vi/Vc X 100)
6 µM Urd
100
80
60
40
20
0
0
9
8
7
6
5
327 µM Urd
100
80
60
40
20
0
0
9
8
7
6
5
-log NBMPR (M)


10 µM dThd
352 µM dThd
Percent transport activity (Vi/Vc X 100)
100
80
60
40
20
0
0
9
8
7
6
5
- log [NBMPR] (M)

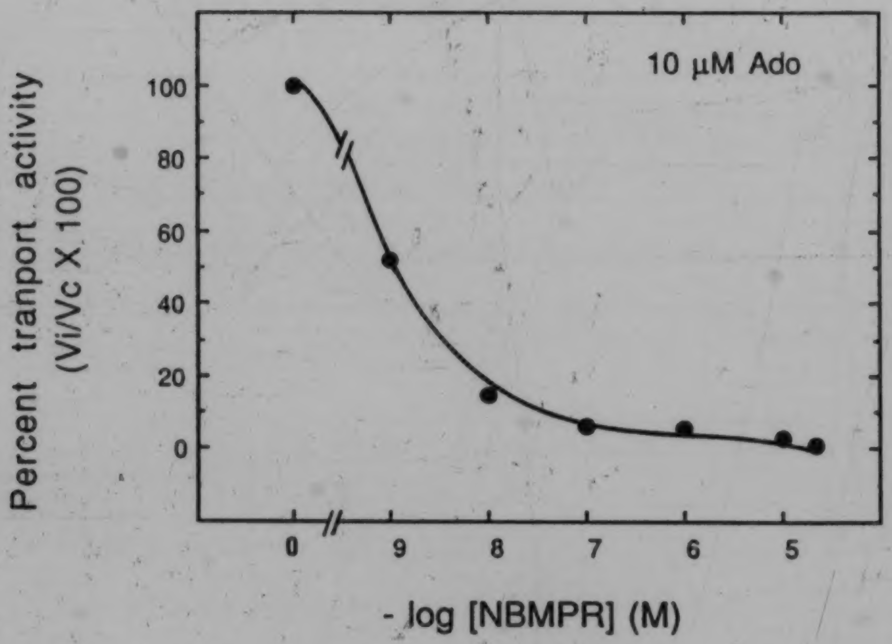

10 μM Ado
Percent tranport activity
(Vi/Vc X 100)
100
80
60
40
20
0
0
9
8
7
6
5
- log [NBMPR] (M)

Table I

IC_{50} values for inhibition by NBMPR of uridine, adenosine and thymidine zero-*trans* influx

Summarized here are data from the experiments presented in Figure 5.

Nucleoside	μM	NBMPR-insensitive transport-activity[α]	IC_{50}[β] (nM)
Uridine	6	10	1.0 ± 0.7 (3)
	327	11	0.8 ± 0.2 (2)
Adenosine	10	9	0.8 (1)
Thymidine	10	7	0.6 (1)
	352	13	0.4 (1)

[α] The proportion (expressed as a percentage) of total transport activity remaining when zero-*trans* influx was estimated in the presence of 1 μM NBMPR.

[β] The IC_{50} value is the concentration of NBMPR that reduced transport rates of the NBMPR-sensitive component to 50% . Values are means (± S.D. or ± M.D.) of the number of experiments indicated in parenthesis or single values in the case of an experiment conducted only once.

for permeation of nucleosides in K562/4 cells. One process exhibited high sensitivity to inhibition by NBMPR with IC_{50} values of 0.4-1.0 nM and the other(s) exhibited low sensitivity with transport still evident at 5 μM. The similar, high sensitivities of inward fluxes of uridine, adenosine and thymidine to NBMPR suggested that the NBMPR-sensitive process consisted of a single system (or similar systems) that mediated influx of nucleosides. The low levels of uptake of nucleosides in the presence of 5 μM NBMPR could have been due to permeation by passive diffusion and/or by one or more mediated transport systems with low (or lacking altogether) sensitivity to NBMPR.

3) *Equilibrium binding of ^{3}H-NBMPR to K562 cells*

In human erythrocytes, which possess a single set of binding sites with high affinity (K_d=0.31 nM) (Jarvis et al 1983) for NBMPR, occupancy of the binding sites by NBMPR is directly correlated with inhibition of transport (Cass et al 1974) and the binding sites are physically associated with polypeptides that mediate equilibrative, NBMPR-sensitive transport (Kwong et al 1988). The high NBMPR-sensitivity of nucleoside influx seen in the experiments of Figure 5 suggested that K562 cells, like erythrocytes, might also have transport inhibitory sites with high affinity to NBMPR. To examine this possibility, binding of ^{3}H-NBMPR to intact K562/4 cells was measured under equilibrium conditions at the same temperature (37°C) used to obtain IC_{50} values for transport inhibition. Binding studies were also conducted at room temperature. Most published studies of NBMPR binding to erythrocytes and various cultured cell types have been conducted at 20-25°C.

Saturable high-affinity binding of ^{3}H-NBMPR to K562/4 cells was demonstrated in the representative equilibrium binding experiment presented in Figure 6. Specificity for interaction of NBMPR with the putative transport-inhibitory sites was indicated by the ability of nitrobenzylthioguanosine (NBTGR), a structural analog, to block binding of ^{3}H-NBMPR. The sites revealed by competition with NBTGR appeared to be of a single type and exhibited high affinity (K_d = 0.5 nM) for NBMPR. In the experiment of Figure 6, 5.8 x 10^5 NBMPR sites/cell were estimated. A component of binding that did not saturate and was evident in the presence of NBTGR apparently represented non-specific binding.

Mean values (± S.D.) for the binding constants from a series of experiments conducted at 22°C and 37°C with K562/4 cells are summarized in Table II. At 22°C, the dissociation constant (K_d) and the maximum binding activity (B_{max}) were, respectively, 0.3 ± 0.1 nM and 4.8 ± 0.9 x 10^5 sites/cell and at 37°C, 0.5 ± 0.0 nM and 5.4 ± 0.5 x 10^5 sites/cell. Although temperature had little if any effect on the number of binding sites, a 2-fold increase in the dissociation constant was observed when the temperature of the binding assay was increased from 22°C to 37°C. This increase was consistent with changes in K_d values with temperature observed in studies of NBMPR binding in Chinese hamster ovary cells and in HeLa cells (Wohlhueter & Plagemann1983, Lauzon & Paterson 1977).

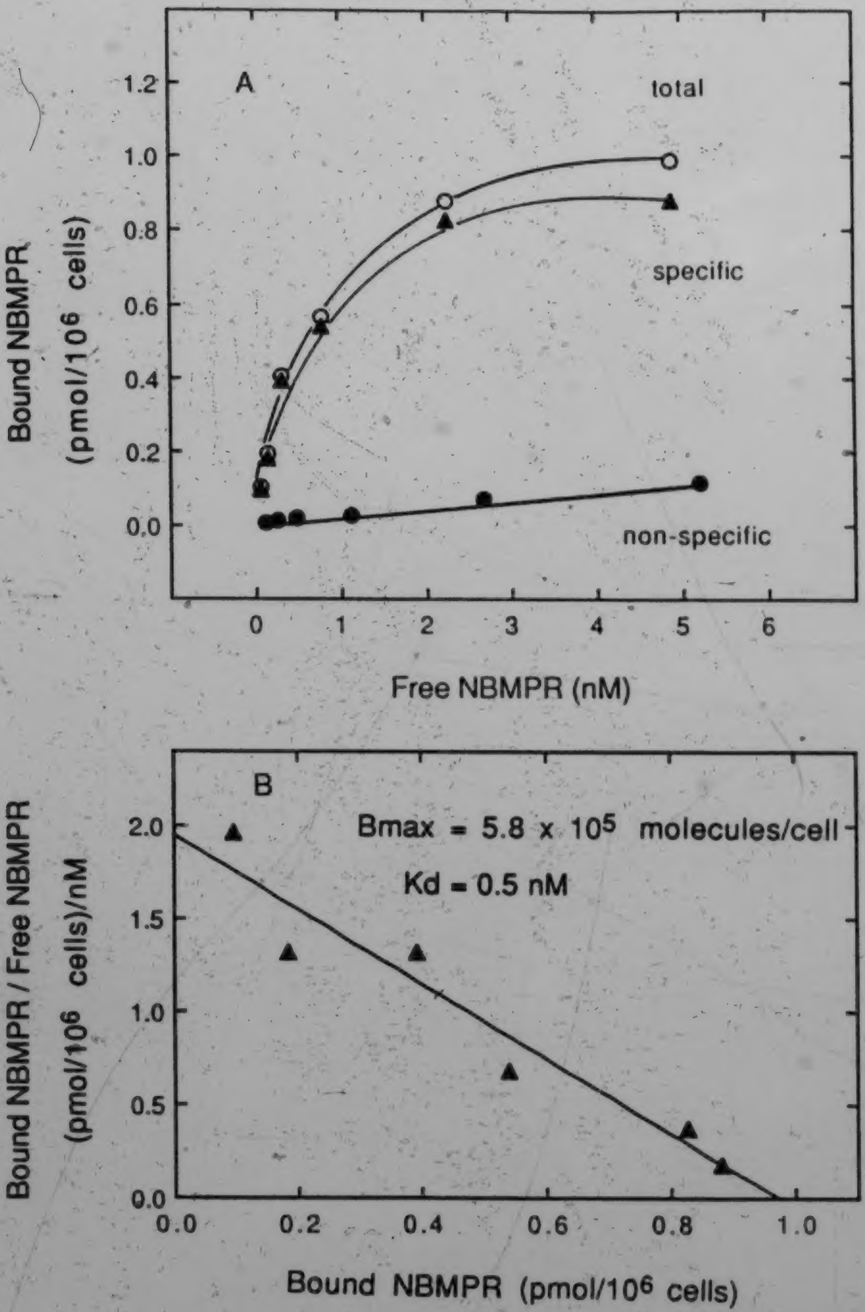
NBMPR binding activity was also examined in the parental K562/ATCC line and two other clonal derivatives, K562/2B1 and

Figure 6

Site-specific binding of ^{3}H-NBMPR to K562/4 cells

Panel A: Equilibrium binding of ^{3}H-NBMPR. Cells (5 x 10^5 cells/assay) were incubated for 30 min at 37°C in TRPMI solution containing ^{3}H-NBMPR (0.1 - 5.0 nM; 4.5 - 222.5 nCi/ml) alone (open circles) or, to determine non-specific binding, also containing 10 µM NBTGR (closed circles). Specifically bound NBMPR (closed triangles) was calculated by subtracting values for non-specific binding from those for total binding. The amounts of cell-associated ^{3}H-NBMPR (y axis) and free ^{3}H-NBMPR at equilibrium (x axis) were determined as described in Chapter II, Section C.6. Presented are mean values of triplicate determinations; S.D. values (not shown) ranged between 0.5-5 % of the mean values.

Panel B: Scatchard analysis. The values for specifically bound NBMPR shown in panel A have been analyzed by the method of Scatchard and binding constants (see Table II) were determined by linear regression analysis. The B_{max} value has been converted from pmol/10^6 cells to the number of molecules/cell.


A
total
specific
non-specific
Bound NBMPR (pmol/10⁶ cells)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
6
Free NBMPR (nM)
B
Bmax = 5.8 x 10⁵ molecules/cell
Kd = 0.5 nM
Bound NBMPR / Free NBMPR (pmol/10⁶ cells)/nM
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0
Bound NBMPR (pmol/10⁶ cells)

K562/1A3, to determine if there were clonal differences in the expression of binding sites. The experiments were conducted at 22°C and the results are presented in Table II. The Bmax values obtained for K562/1A3, K562/2B1 and K562/ATCC cells were slighlty lower than the values obtained for K562/4 cells. Although similar Kd values (0.3 nM) were obtained for the K562/2B1, K562/1A3 and K562/4 clones, a somewhat lower value (0.1 nM) was obtained for the K562/ATCC line.

Thus, K562 cells express a large number of sites to which NBMPR binds with high affinity, with Kd values within the range (0.1-2.0 nM) previously observed for erythrocytes and several other types of cultured cells (Paterson et al 1987, Jarvis et al 1982, Cass et al 1979). There was a good correlation between the Kd value (0.5 nM) obtained at 37°C (Table II) and the IC_{50} values (0.4-1.0 nM) for NBMPR inhibition of nucleoside transport activity (Table I). This correspondence between the Kd and IC_{50} values suggested that, at NBMPR concentrations lower than 10 nM, the concentration-effect curves of Figure 5 for transport inhibition by NBMPR describe the effects of NBMPR occupancy of the high-affinity binding sites.

4) *Kinetics of uridine and adenosine zero-trans influx*

The kinetic behavior of the nucleoside transport system of human erythrocytes, which is both equilibrative and NBMPR sensitive, is consistent with that proposed for a single carrier mechanism (Cabantchick & Ginsburg 1977). One of the features of this process is saturability of permeation rates as a function of nucleoside concentration. Studies of the kinetic properties of zero-*trans* influx of

Table II

NBMPR binding constants for different clones of K562 cells

Equilibrium binding constants were determined as described in Figure 6 at room temperature (about 22°C) or at 37°C. When cells were harvested, they were actively proliferating at culture densities that ranged from 2.4 x 10^5 to 4.6 x 10^5 cells/ml. The number of determinations are indicated in parentheses and, where more than one experiment was conducted, values are means (± S.D. or M.D.)

K562 sublines	Temperature (°C)	K_d (nM)	B_{max} (sites/cell) x 10^5
K562/4	22 (7)	0.3 ± 0.1	4.8 ± 0.9
K562/4	37 (2)	0.5 ± 0.0	5.4 ± 0.5
K562/2B1	22 (1)	0.3	3.1
K562/1A3	22 (1)	0.3	2.8
K562/ATCC	22 (1)	0.1	3.7

nucleosides in cells that lack sensitivity to NBMPR have indicated that some of the NBMPR-insensitive systems are also equilibrative processess with saturable kinetics and broad substrate specificities (Belt 1983, Jarvis & Young 1980, Plagemann & Wohlhueter 1985a).

To further characterize nucleoside transport in K562/4 cells, the kinetic properties of uridine and adenosine zero-*trans* influx were investigated under conditions that allowed separation of the transport process into NBMPR-sensitive and NBMPR-insensitive components. This was accomplished by determining the concentration dependance of transport velocities in the absence of NBMPR and in the presence of a concentration of NBMPR that saturated the high-affinity binding sites. Transport velocities obtained in the presence of NBMPR represented the NBMPR-insensitive component, and the arithmetic difference between total velocities and those obtained in the presence of NBMPR represented the NBMPR-sensitive component.

Values for zero-*trans* influx of uridine and adenosine in either the absence or presence of NBMPR were obtained as described in Chapter II, Section C.3 from 3-sec time courses of cellular uptake for at least six different permeant concentrations (Figures 7, 8, 9, 10). For both nucleosides, uptake approached equilibrium rapidly, especially at the lower concentrations (insets, Figures 7, 9). However, since uptake at time "0" was determined for each time course, the 3-sec interval was sufficiently short to allow estimation of initial rates, even at the lowest concentrations of uridine or adenosine.

The values for extracellular space calculated from the pellet contents of radioactivity at time "0" in the time courses obtained in the absence of NBMPR (Figures 7 and 9) were similar to the values predicted from the size of the ^{14}C-sucrose space. Furthermore, it can be seen from Figure 11 that time "0" values for the ^{3}H-uridine and ^{3}H-adenosine contents of cell pellets were linearly related to concentration, even at mM concentrations. This proportionality, together with the equivalence of the ^{14}C-sucrose and the ^{3}H-nucleoside spaces, suggested that the association of ^{3}H-labelled nucleosides with cell pellets at time "0" was due primarily to trapping in the extracellular space during centrifugation.

Influx of both uridine and adenosine, either in the presence or absence of NBMPR, exhibited saturation kinetics (Figure 12). The NBMPR-sensitive components of uridine and adenosine influx, which were obtained by subtracting fluxes in the presence from those in the absence of NBMPR, were also saturable. The hyperbolic relationships of rate versus concentration could be described by the Michaelis-Menten equation as is indicated by the good fitting of the data to the linear transformations (S_1/v_{12} versus S_1, v_{12} versus v_{12}/S_1) shown in Figures 13 and 14. It was obvious, even from Figure 12, that the NBMPR-sensitive and NBMPR-insensitive components of influx exhibited different kinetic properties.

The kinetic parameters, K_m and V_{max}, were determined from linear transformations of the Michaelis Menten equation: $v = \frac{V_{max}S}{(K_m+S)}$. Graphic representations of these transformations (S_1/v_{12} versus S_1, v_{12}/S_1 versus v_{12}) using the data of the experiment of Figure 12 are shown in Figure 13 for uridine and Figure 14 for adenosine. A single example of a direct linear plot, using the results obtained when uridine transport rates were assayed in the absence of NBMPR, is shown in Figure 15 (similar plots were obtained when adenosine transport data were analysed according to this method). Although the S_1/v_{12} versus S_1 plot emphasizes data obtained at relatively high concentrations and the v_{12}/S_1 versus v_{12} plot emphasizes the opposite, the kinetic parameters derived from these plots were similar and correlated well with the parameters derived from the direct linear plots. The correspondence in kinetic parameters obtained with these three analytical procedures is shown in Table III, which presents results from several experiments that measured transport of uridine and adenosine in the absence of NBMPR. It appeared that the variances in rate measurements were relatively constant throughout the concentration range used for the kinetic analyses.

Table IV summarizes the results from all the kinetic studies conducted with uridine and adenosine, including those of Figure 12. Kinetic parameters for uridine (Table IV-A) and adenosine (Table IV-B) were calculated for the total transport process and the NBMPR-sensitive and NBMPR-insensitive components of the transport process. The NBMPR-sensitive components were identified by subtraction of fluxes determined in the presence of NBMPR from total fluxes. Although total influx represented the combined activities of at least two transport processes, evaluation of its kinetic properties provided an indication of the overall transport capacity of cells at various permeant concentrations.

For both uridine and adenosine, NBMPR-sensitive and NBMPR-insensitive influx differed in permeant affinities (K_m values), maximum translocation capacities (V_{max} values), and limiting permeabilities[2] (π_{12}^{zt}) values. NBMPR-sensitive influx of uridine exhibited a 5-fold higher affinity and a 3-fold higher maximal velocity than NBMPR-insensitive influx. The limiting permeability for uridine transport by the NBMPR-sensitive process was 15-fold greater than that of the NBMPR-insensitive process, indicating greater efficiency, especially at low uridine concentrations, of the NBMPR-sensitive process. Similarly, NBMPR-sensitive influx of adenosine, exhibited a 3-fold higher affinity and a 5-fold higher maximal velocity than NBMPR-insensitive influx. At very low (sub-Km) concentrations of adenosine the difference in efficiency was large, with the NBMPR-insensitive process contributing only about 10% of the total transport activity.

For uridine, NBMPR-insensitive transport comprised 7-13% of total influx when concentrations were between 11 and 550 µM and increased to 26% at concentrations >1.5 mM. For adenosine, NBMPR-insensitive transport comprised 5-12% of total influx at concentrations <100 µM and 18-25% of total influx at concentrations >100 µM. Thus, at low concentrations it appeared that almost all of the intracellularly accumulated ^{3}H-uridine or ^{3}H-adenosine entered cells via the NBMPR-sensitive route. The NBMPR-insensitive route, with its low affinity and low capacity, would be expected to assume more importance at concentrations greater than the Km for total transport, although it would probably not exceed 25% of total transport capacity.

---

[2] Limiting permeability (π_{12}^{zt}) represents the efficiency (i.e., the fraction of permeant transported / unit of time) of translocation of nucleosides in one direction in the absence of backflux or any metabolic trapping.

Figure 7

Time courses of uridine zero-*trans* influx

Suspensions of actively proliferating K562/4 cells in TRPMI medium (1.0×10^7-1.5×10^7 cells/ml) were used to assay cellular uptake during "0", 1, 2 and 3 sec of exposure to ^{3}H-uridine (final concentrations 10-5,002 µM, 35-37 µCi/ml). Cells were prepared from 4 consecutive harvests and cells of each harvest were used to measure transport of 3-4 concentrations. Uptake was assayed by the "inhibitor-oil stop" method as described in Chapter II, Section C2. Time "0" values were obtained by (i) addition of dilazep solution to the ^{3}H-permeant solution, then (ii) addition of cells and immediate centrifugation through the oil layer. The data presented are means of 2 or 3 determinations and are from a single representative experiment. The uptake curves for 10 and 28 µM are reproduced with an expanded scale in the inset.

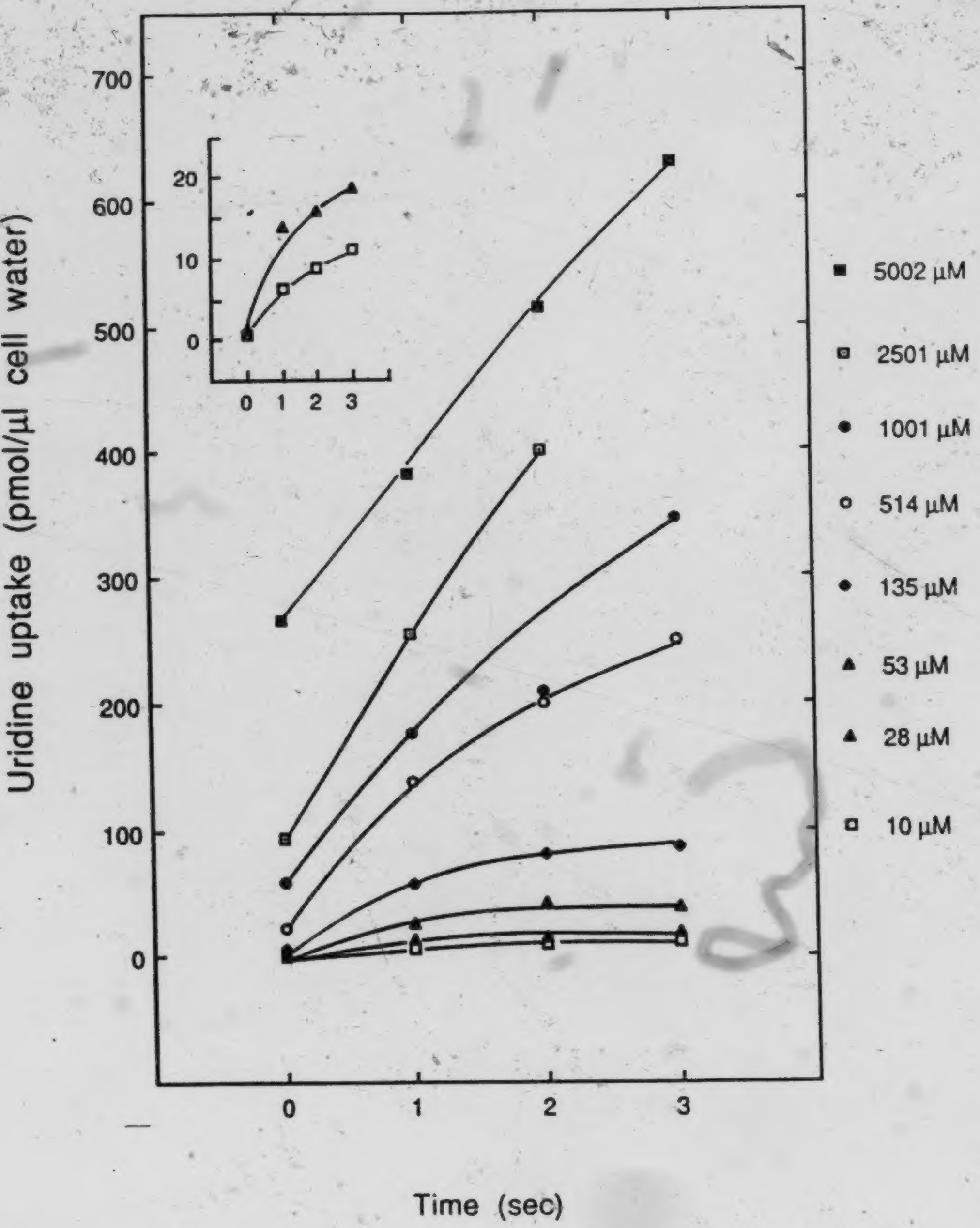

Uridine uptake (pmol/µl cell water)
700
600
500
400
300
200
100
0
0
1
2
3
Time (sec)
20
10
0
0
1
2
3
■ 5002 µM
▣ 2501 µM
● 1001 µM
○ 514 µM
● 135 µM
▲ 53 µM
▲ 28 µM
□ 10 µM

Figure 8

Time courses of uridine zero-*trans* influx in the presence of 0.1 μM NBMPR

Suspensions of actively proliferating K562/4 cells (1.0 x 10^7 to 1.5 x 10^7 cells/ml) were incubated at 37°C for at least 15 min in TRPMI medium containing 0.1 μM NBMPR and were then used to assay cellular uptake during "0", 1, 2, and 3 sec of exposure to ^{3}H-uridine (final concentrations 28-5,002 μM, 35-37 μCi/ml) and 0.1 μM NBMPR. Cells were harvested and transport assays were conducted as described in Figure 8. The data presented are means of 2 or 3 determinations and are from a single representative experiment. The uptake curves for 28 and 53 μM are reproduced with an expanded scale in the inset.

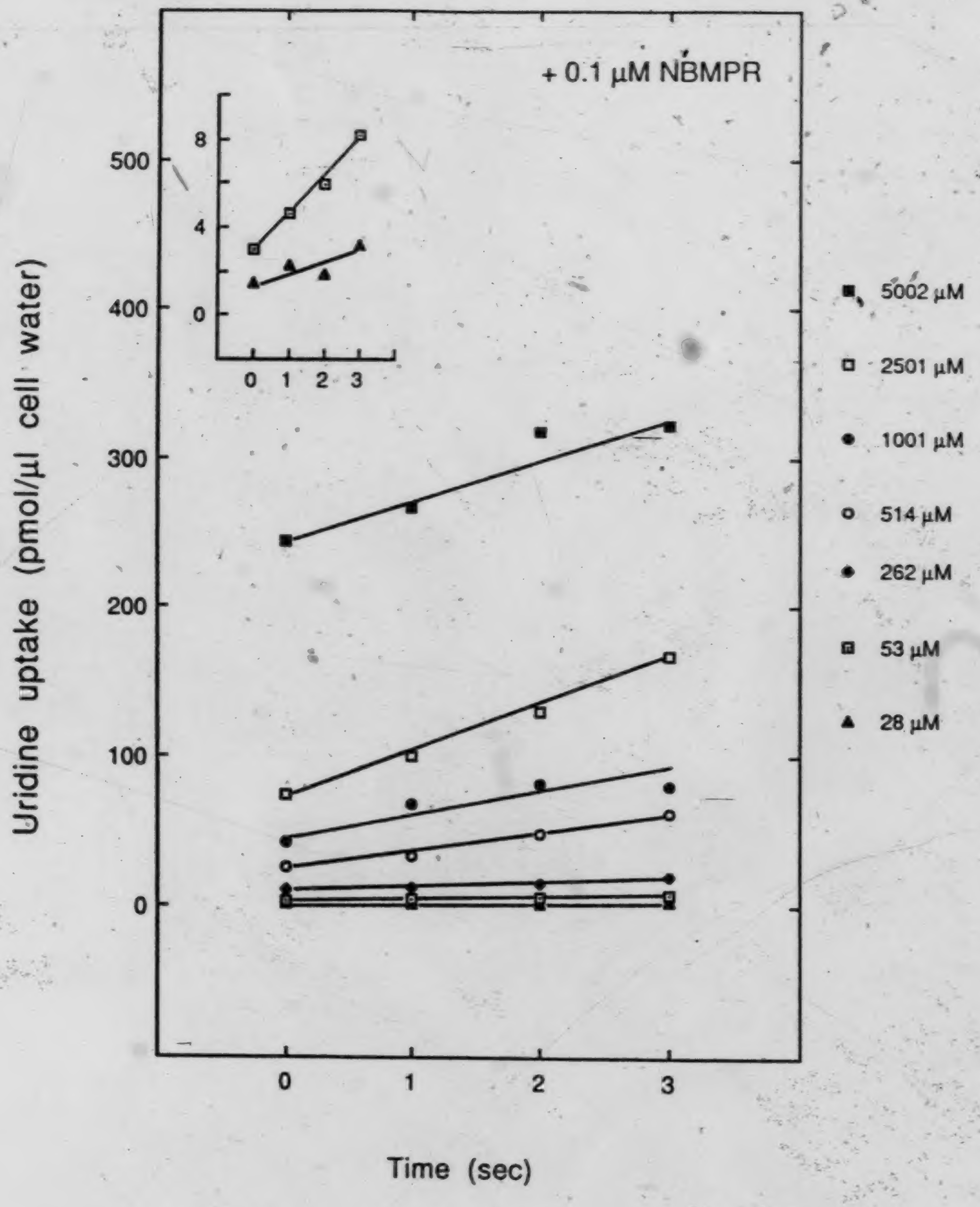

+ 0.1 µM NBMPR
Uridine uptake (pmol/µl cell water)
500
400
300
200
100
0
0
1
2
3
Time (sec)
8
4
0
0
1
2
3
■ 5002 µM
□ 2501 µM
● 1001 µM
○ 514 µM
● 262 µM
▣ 53 µM
▲ 28 µM

Figure 9

Time courses of adenosine zero-*trans* influx. Suspensions of actively proliferating K562/4 cells in TRPMI medium (1.0×10^7-1.5×10^7 cells/ml) were used to assay cellular uptake during "0", 1, 2, and 3 sec of exposure to ^{3}H-adenosine (final concentrations 5-500 µM, 34 µCi/ml). Cells were prepared from 2 consecutive harvests and cells of each harvest were used to measure transport of 3-4 concentrations. Transport assays were conducted as described in Figure 8. The data presented are means of 2 or 3 determinations and are from a single representative experiment. The uptake curves for 5 and 27 µM are reproduced with an expanded scale in the inset.

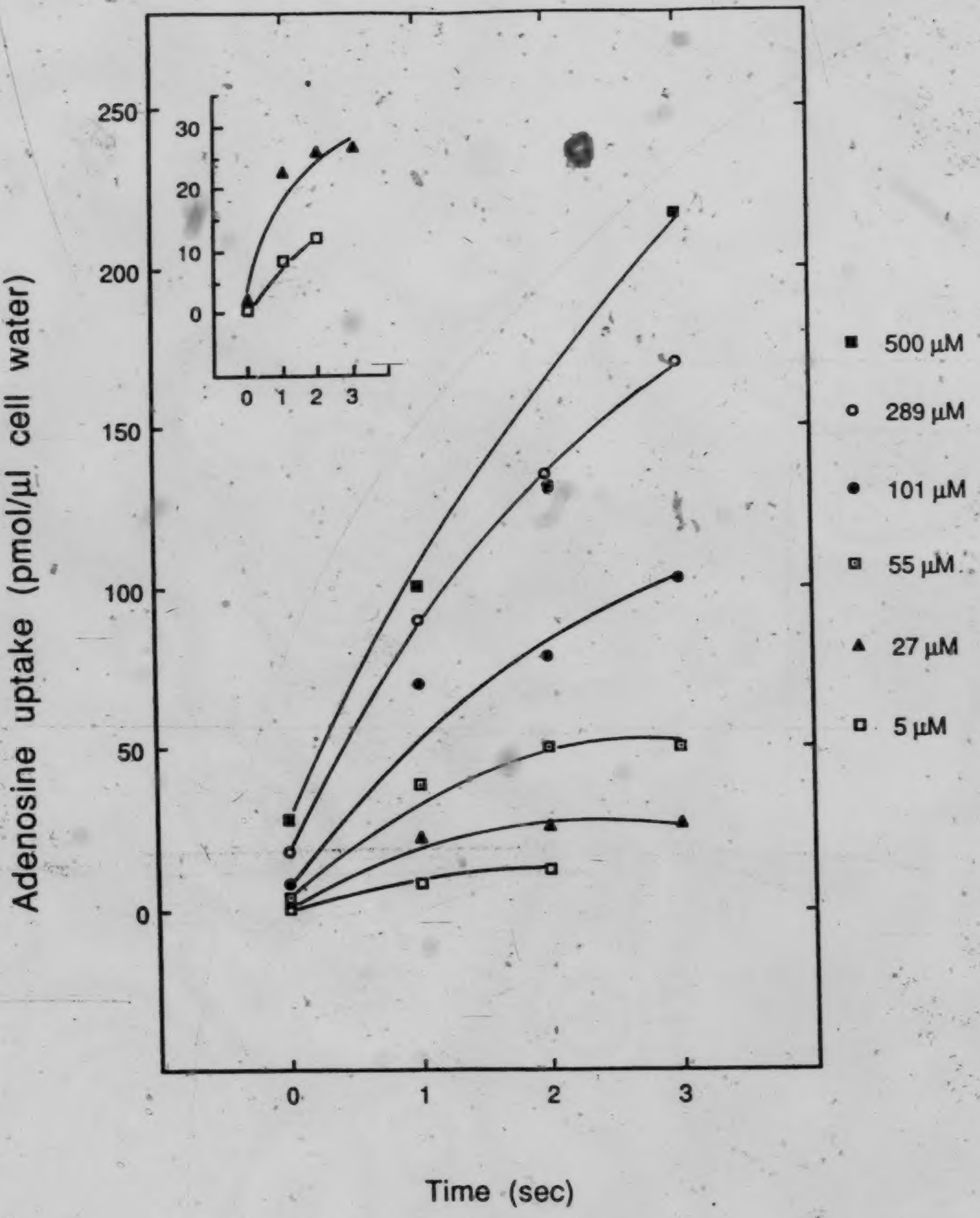

Adenosine uptake (pmol/µl cell water)
250
200
150
100
50
0
0
1
2
3
Time (sec)
30
20
10
0
0
1
2
3
■ 500 µM
○ 289 µM
● 101 µM
▣ 55 µM
▲ 27 µM
□ 5 µM

Figure 10

Time courses of adenosine zero-*trans* influx in the presence of 0.5 μM NBMPR

Suspensions of actively proliferating K562/4 cells (1.0 x 10^7 to 1.5 x 10^7 cells/ml) were incubated at 37°C for at least 15 min and were then used to assay cellular uptake during "0", 1, 2 and 3 sec of exposure to ^{3}H-adenosine (final concentrations 5-500 μM, 34 μCi/ml) and 0.5 μM NBMPR. Cells were prepared from 2 consecutive harvests and cells of each harvest were used to measure transport of 3-4 concentrations as described in Figure 8. The data presented are means of [illegible] or 3 determinations and are from a single representative experiment.

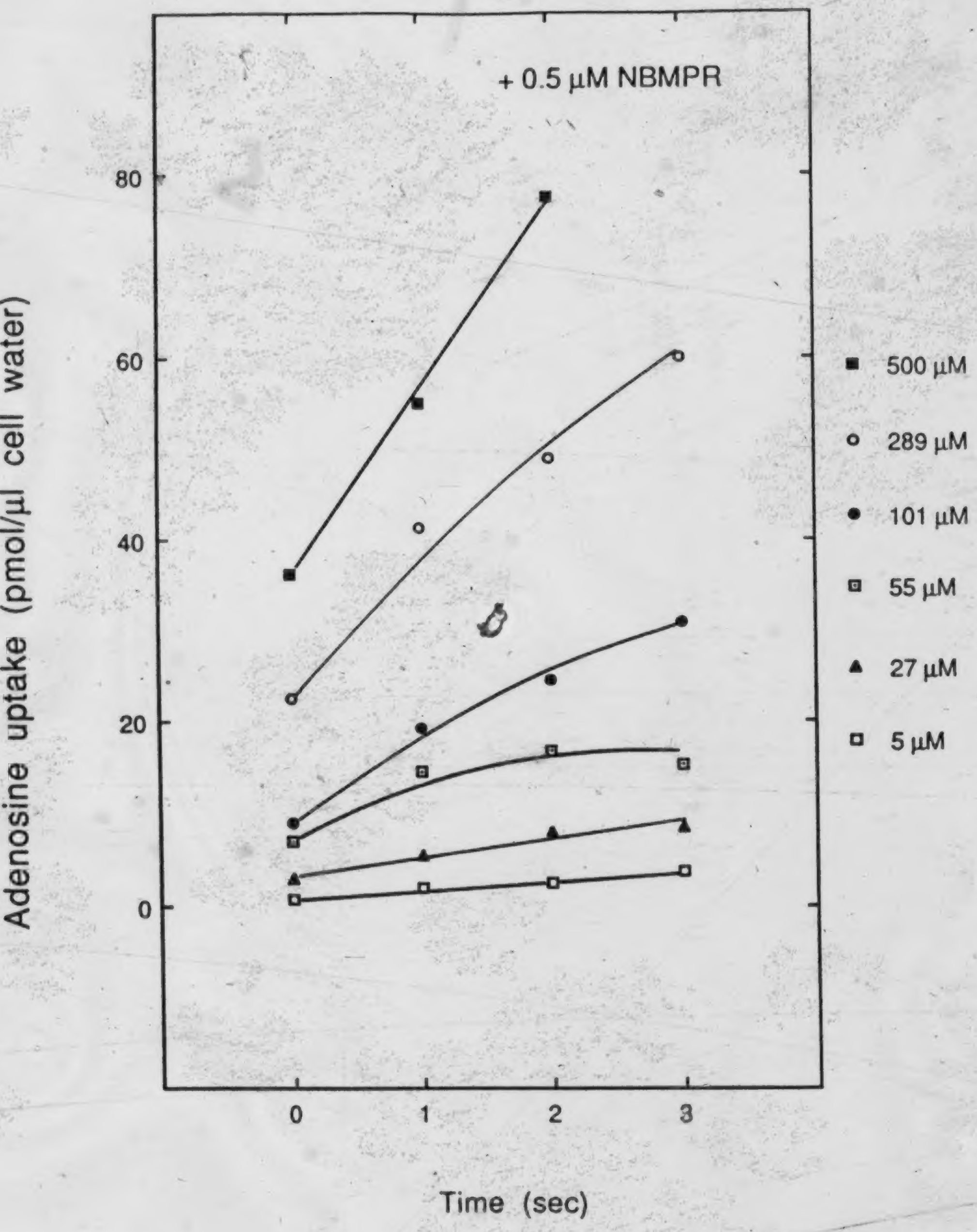
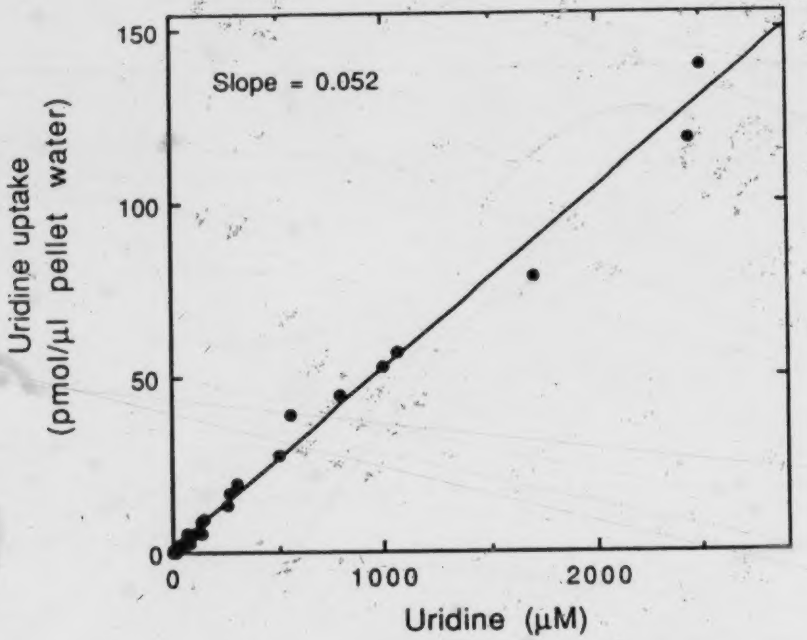

+ 0.5 μM NBMPR
Adenosine uptake (pmol/μl cell water)
80
60
40
20
0
0
1
2
3
Time (sec)
■ 500 μM
○ 289 μM
● 101 μM
▣ 55 μM
▲ 27 μM
□ 5 μM

Figure 11

Uridine and adenosine associated with pellets at time "0" as a function of extracellular concentration

Uptake of ^{3}H-uridine or ^{3}H-adenosine at time "0" was obtained by (i) addition of dilazep in the permeant containing transport medium, and then (ii) addition of cells and immediate centrifugation through oil. The data presented in these graphs are summarized from 3 or 2 experiments, respectively, for uridine (Panel A) and adenosine (Panel B). Points are means of 3 up to 6 determinations.


Slope = 0.052
Uridine uptake (pmol/µl pellet water)
150
100
50
0
1000
2000
Uridine (µM)

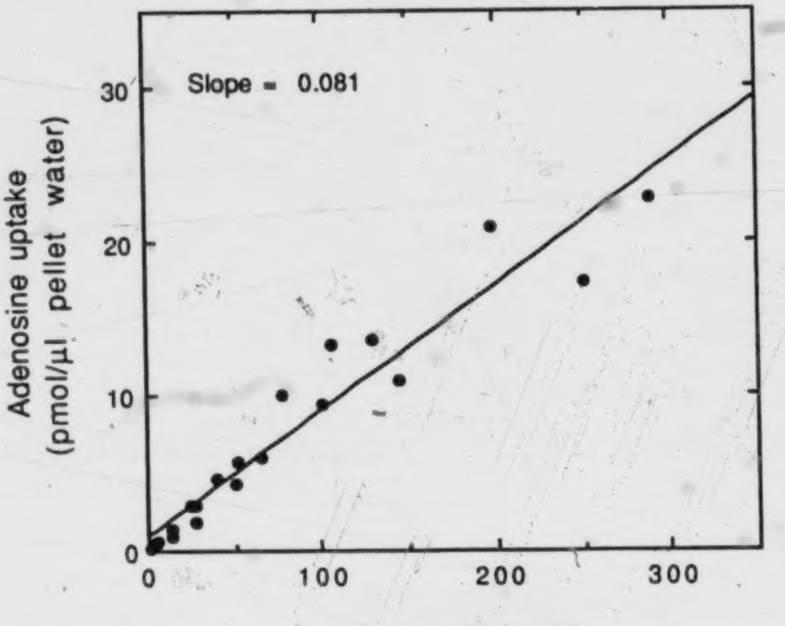
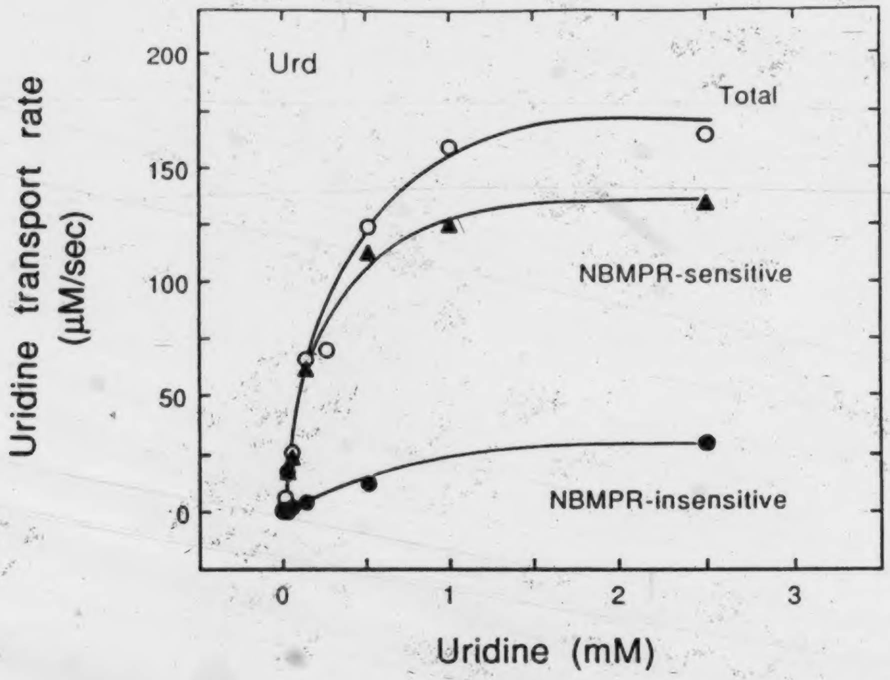

Slope = 0.081
Adenosine uptake (pmol/µl pellet water)
30
20
10
0
100
200
300
Adenosine (µM)

Figure 12

Concentration dependance of uridine and adenosine zero-*trans* influx

Transport rates were estimated as the first order terms of straight lines or 2nd order polynomial equations describing curves fitted to the time courses of cellular uptake of ^{3}H-uridine alone (Figure 8) or in the presence of 0.1 µM NBMPR (Figure 9) and of ^{3}H-adenosine alone (Figure 10) or in the presence of 0.5 µM NBMPR (Figure 11). Plotted are transport rates in the absence of NBMPR (closed circles), transport rates in the presence of NBMPR (open circles), and the arithmetic difference between transport rates in the absence and presence of NBMPR (closed triangles).


Urd
Total
NBMPR-sensitive
NBMPR-insensitive
Uridine transport rate (µM/sec)
200
150
100
50
0
0
1
2
3
Uridine (mM)

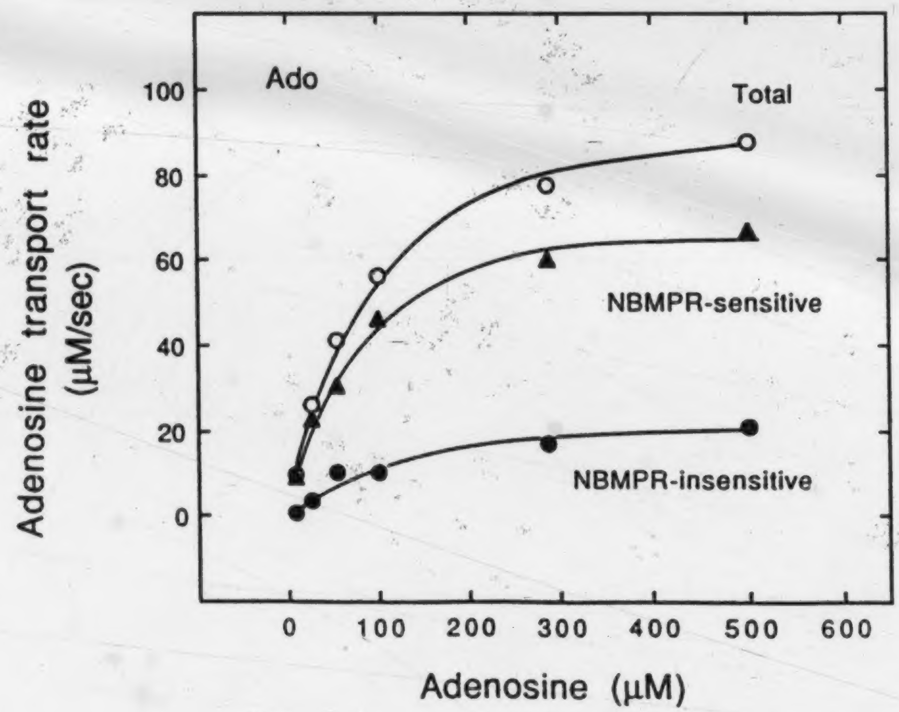

Ado
Total
NBMPR-sensitive
NBMPR-insensitive
Adenosine transport rate (µM/sec)
100
80
60
40
20
0
0
100
200
300
400
500
600
Adenosine (µM)

Figure 13

Uridine zero-*trans* influx kinetics

Data from the experiment of Figure 13A are presented in the linear forms S_1/v_{12} versus S_1 and v_{12} versus v_{12}/S_1. Linear squares analysis of these plots yielded kinetic parameters for total uridine transport activity determined in the absence of NBMPR; NBMPR-sensitive uridine transport estimated by subtracting rates obtained in the presence of 0.1 µM NBMPR from those obtained in the absence of NBMPR; and NBMPR-insensitive transport determined in the presence of 0.1 µM NBMPR.

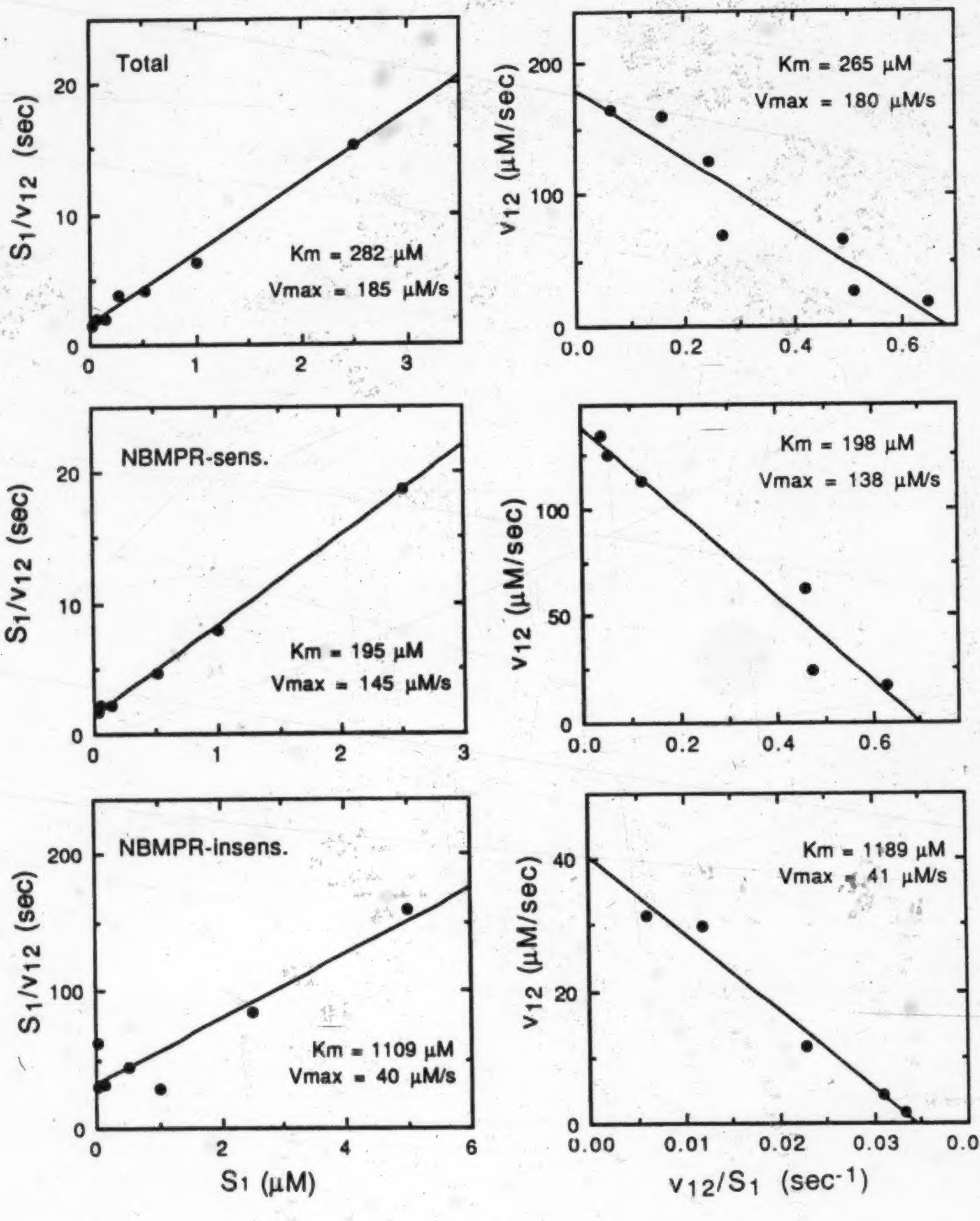

Total
Km = 282 µM
Vmax = 185 µM/s
Km = 265 µM
Vmax = 180 µM/s
NBMPR-sens.
Km = 195 µM
Vmax = 145 µM/s
Km = 198 µM
Vmax = 138 µM/s
NBMPR-insens.
Km = 1109 µM
Vmax = 40 µM/s
Km = 1189 µM
Vmax = 41 µM/s
S1/v12 (sec)
v12 (µM/sec)
S1 (µM)
v12/S1 (sec-1)
0
10
20
100
200
1
2
3
4
5
6
0.0
0.2
0.4
0.6
50
40
0.00
0.01
0.02
0.03
0.04

Figure 14

Adenosine zero-*trans* influx kinetics

Data from the experiment of Figure 13B are presented in the linear forms S_1/v_{12} versus S_1 and v_{12} versus v_{12}/S_1. Linear squares analysis of these plots yielded kinetic parmeters for total uridine transport activity determined in the absence of NBMPR; NBMPR-sensitive uridine transport estimated by subtracting rates obtained in the presence of 0.5 µM NBMPR from those obtained in the absence of NBMPR; and NBMPR-insensitive transport determined in the presence of 0.5 µM NBMPR.

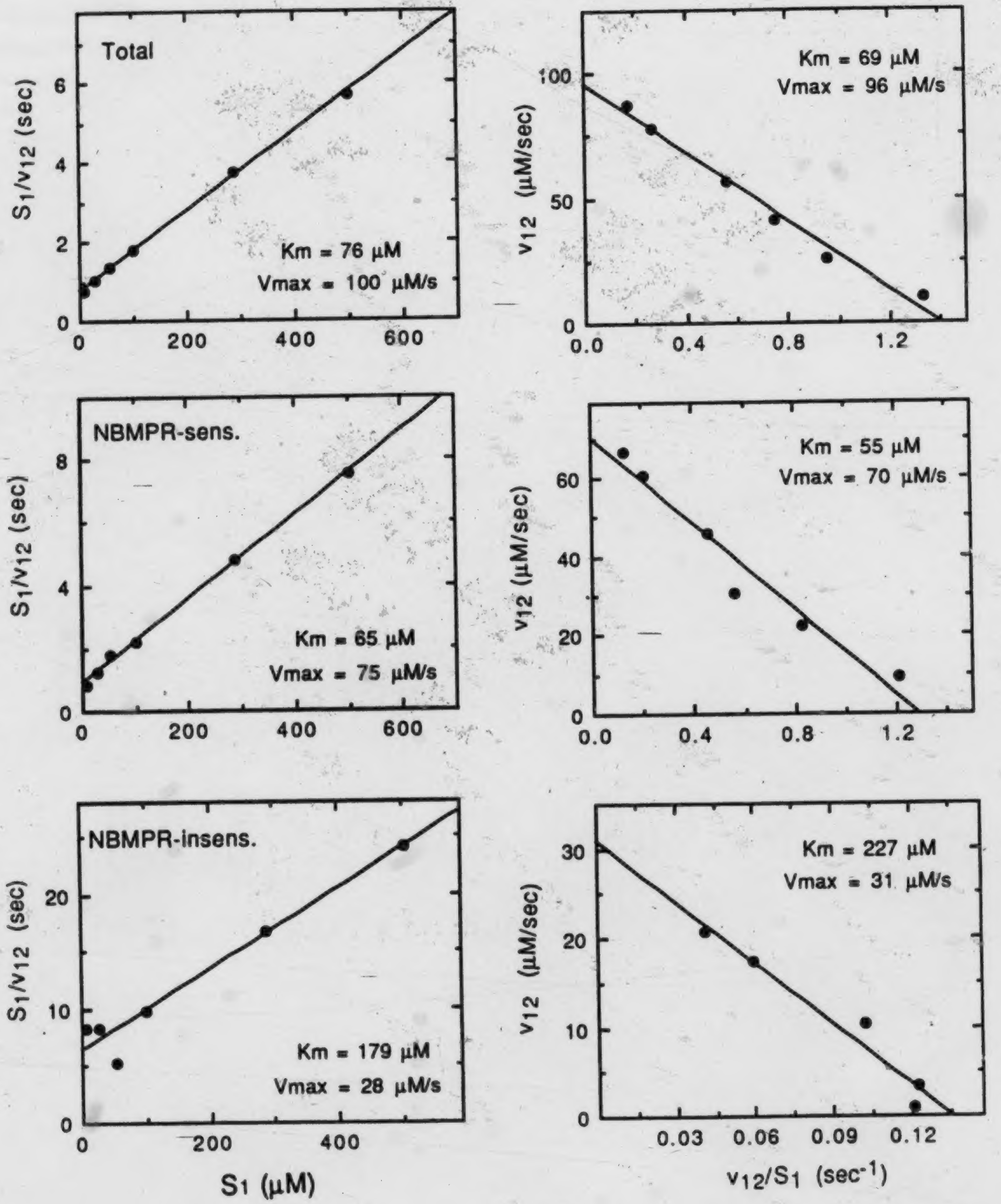

Total
S_1/v_{12} (sec)
Km = 76 µM
Vmax = 100 µM/s
0
2
4
6
0
200
400
600
Km = 69 µM
Vmax = 96 µM/s
v_{12} (µM/sec)
0
50
100
0.0
0.4
0.8
1.2
NBMPR-sens.
S_1/v_{12} (sec)
Km = 65 µM
Vmax = 75 µM/s
0
4
8
0
200
400
600
Km = 55 µM
Vmax = 70 µM/s
v_{12} (µM/sec)
0
20
40
60
0.0
0.4
0.8
1.2
NBMPR-insens.
S_1/v_{12} (sec)
Km = 179 µM
Vmax = 28 µM/s
0
10
20
0
200
400
S_1 (µM)
Km = 227 µM
Vmax = 31 µM/s
v_{12} (µM/sec)
0
10
20
30
0.03
0.06
0.09
0.12
v_{12}/S_1 (sec^{-1})

Figure 15

Direct linear plot of ^{3}H-uridine zero-*trans* influx

Plotted are values from the experiment of Figure 13 for ^{3}H-uridine influx in the absence of NBMPR. For each rate value, a straight line defined by the concentration of ^{3}H-uridine and the observed rate was drawn. The coordinates of the intersection points for a set of 7 lines (for 7 rate determinations) gave a set of 16 pairs of Km and Vmax values. The median Km and Vmax values for this group of kinetic parameters were 255 µM and 185 µM/sec, respectively.

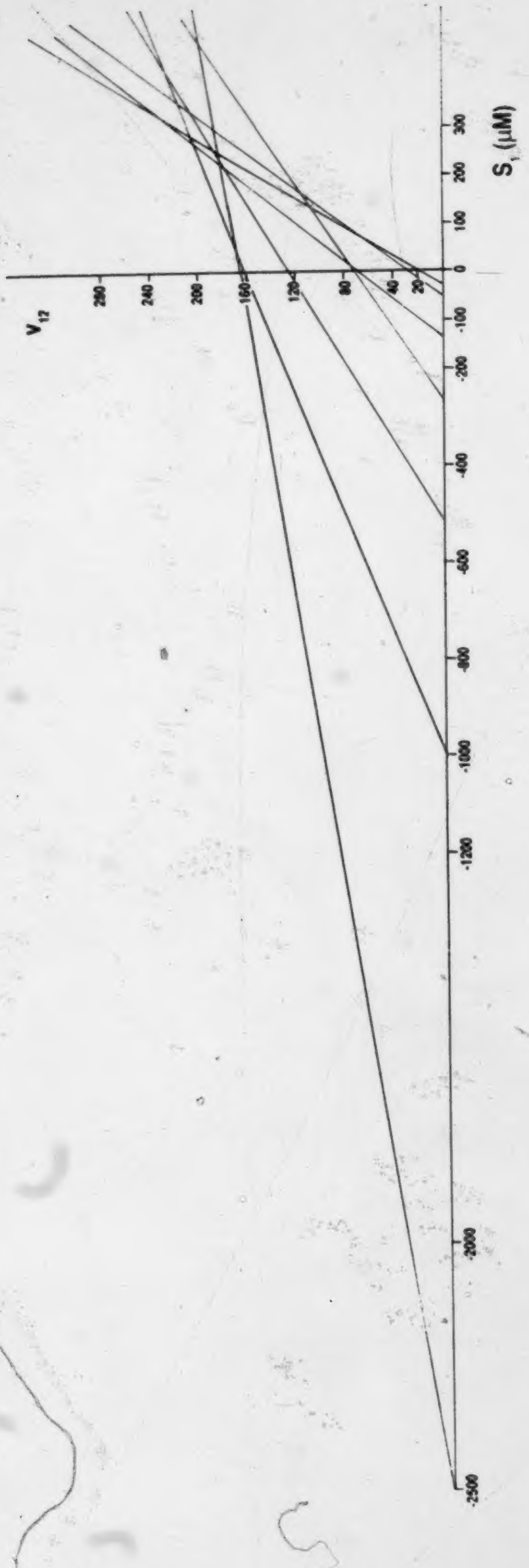

v
12
280
240
200
160
120
80
40
20
S
1
 (μM)
300
200
100
0
-100
-200
-400
-600
-800
-1000
-1200
-2000
-2500

Table III

Comparison of three analytical methods for derivation of kinetic parameters of zero-trans influx of uridine and adenosine measured in the absence of NBMPR.

Kinetic parameters for total ^{3}H-uridine and ^{3}H-adenosine zero-*trans* influx were derived by linear regression analysis of plots of v_{12} versus v_{12}/S_1 and S_1/v_{12} versus S_1 and by the direct linear plot method, from three (uridine) or two (adenosine) experiments, including the ones of Figure 13. Influx of at least 6 concentrations, ranging from 0.005-5 mM (uridine) or 2-500 μM (adenosine) was examined in each experiment.

Table III

Parameter	Method		
	v_{12} against v_{12}/S_1	S_1/v_{12} against S_1	direct linear plot
Vmax (µM/sec)			
uridine	162 ± 19	180 ± 18	161 ± 22
adenosine	96 ± 0.5	95 ± 6	93 ± 5
Km (µM)			
uridine	254 ± 49	314 ± 78	204 ± 59
adenosine	78 ± 10	74 ± 3	72 ± 5
$\pi^{zt}_{12} = \frac{Vmax}{Km}$ (sec-1)			
uridine	0.66 ± 0.15	0.60 ± 0.18	0.71 ± 0.19
adenosine	1.27 ± 0.13	1.23 ± 0.04	1.29 ± 0.15

Table IV-A

Kinetic parameters for uridine zero-*trans* influx

Inward fluxes of uridine at 37°C were estimated as described in Figure 13 from time courses of uptake of ^{3}H-uridine alone or in the presence of NBMPR. Influx of at least 6 concentrations, ranging from 0.005-0.5 mM was examined in each of the three experiments presented. Kinetic parameters are mean values (± S.D.) of those derived by linear regression analysis of plots of v_{12} versus v_{12}/S_1 and S_1/v_{12} against S_1 and by the direct linear plot method. The mean values (± S.D.) for all three experiments are presented in bold type.

Total influx			NBMPR-sensitive influx			NBMPR-insensitive influx		
Km (µM)	Vmax (µM/sec)	π^{zt}_{12} (sec^{-1})	Km (µM)	Vmax (µM/sec)	π^{zt}_{12} (sec^{-1})	Km (µM)	Vmax (µM/sec)	π^{zt}_{12} (sec^{-1})
214 ± 39	173 ± 19	0.81 ± 0.07						
267 ± 62*	183 ± 3*	0.68 ± 0.03*	195 ± 3*	147 ± 10*	0.75 ± 0.05*	1120 ± 63*	40 ± 1*	0.04 ± 0.00*
320 ± 73	148 ± 13	0.47 ± 0.06	262 ± 20	119 + 3	0.45 + 0.03	1033 ± 334	40 ± 7	0.04 ± 0.01
267±63	**168±19**	**0.66±0.1**	**229±39**	**133±17**	**0.60±0.17**	**1077±220**	**40±5**	**0.04±0.00**

* Values are from experiment presented in Figure 13.

Table IV-B

Kinetic parameters for adenosine zero-*trans* influx

Inward fluxes of adenosine at 37^{o}C were estimated as described in Figure 13 from time courses of uptake of ^{3}H-adenosine alone or in the presence of NBMPR. Influx of at least 6 concentrations, ranging form 2-500 μM was examined in each of the two experiments presented. Kinetic parameters are mean values (± S.D.) of those derived by linear regression analysis of plots of v_{12} versus v_{12}/S_1 and S_1/v_{12} versus S_1 and by the direct linear plot method. The mean values (± S.D.) for both experiments are presented in bold type.

Total influx			NBMPR-sensitive influx			NBMPR-insensitive influx		
Km (μM)	Vmax (μM/sec)	π^{zt}_{12} (sec^{-1})	Km (μM)	Vmax (μM/sec)	π^{zt}_{12} (sec^{-1})	Km (μM)	Vmax (μM/sec)	π^{zt}_{12} (sec^{-1})
71 ± 5*	98 ± 2*	1.38 ± 0.07*	61 ± 6*	73 ± 3*	1.21 ± 0.7*	193 ± 29*	29 ± 2*	0.15±0.13*
79 ± 9	91 ± 5	1.16 ± 0.08	55 ± 10	67 ± 5	1.24 ± 0.12	175± 29	15 ± 2	0.09±0.01
75±5	**94±5**	**1.27±0.14**	**61 ±9**	**70±5**	**1.22±0.09**	**186 ±31**	**23 ±8**	**0.12±0.03**

* Values are from experiment presented in Figure 13.

Thus, the two nucleoside transport processes of K562/4 cells exhibited differences in their apparent affinities for uridine and adenosine and in their capacities to transport these nucleosides across the plasma membrane. The NBMPR-sensitive process, with its high affinity and high capacity, was the major functional transport system at low uridine and adenosine concentrations. Influx of uridine and adenosine by the NBMPR-insensitive route was < 25% of total transport, even at concentrations that were greater than the K_m value for the total transport process.

5) Formycin B uptake in K562/4 cells

Formycin B is the C analogue of inosine and is not susceptible to cleavage by purine nucleoside phosphorylase (Sheen et al 1968). In addition, most animal tissues lack the ability to convert inosine to IMP and therefore probably cannot convert formycin B to the monophosphate derivative. Formycin B is not catabolized by human erythrocytes or mouse P388 leukemia cells and is phosphorylated, if at all, very inefficiently (Plagemann & Woffendin 1989, Sheen et al 1968). The relative inertness of formycin B allows measurements of its transport uncomplicated by metabolic conversions. Formycin B has been used extensively as a non-metabolized permeant to study transport in cells that metabolize nucleosides (Vijayalakshmi & Belt 1988, Dagnino 1988, Plageman & Woffendin 1988).

To confirm the equilibrative nature of the NBMPR-sensitive transporter and explore the nature of the NBMPR-insensitive process(es), formycin B influx was examined in K562/4 cells in the absence or presence of NBMPR concentrations (0.1, 15 μM) in excess of those required to saturate the high-affinity sites (Figure 16). In the absence of NBMPR, intracellular formycin B reached steady-state values within < 30 sec and, in the presence of 0.1 μM NBMPR, within 1 min. For both conditions, the intracellular levels of formycin B remained constant for up to 5 min, at concentrations that were equimolar or slightly higher than extracellular concentrations. These results demonstrated equilibration of formycin B by both the NBMPR-sensitive and NBMPR insensitive transport processes.

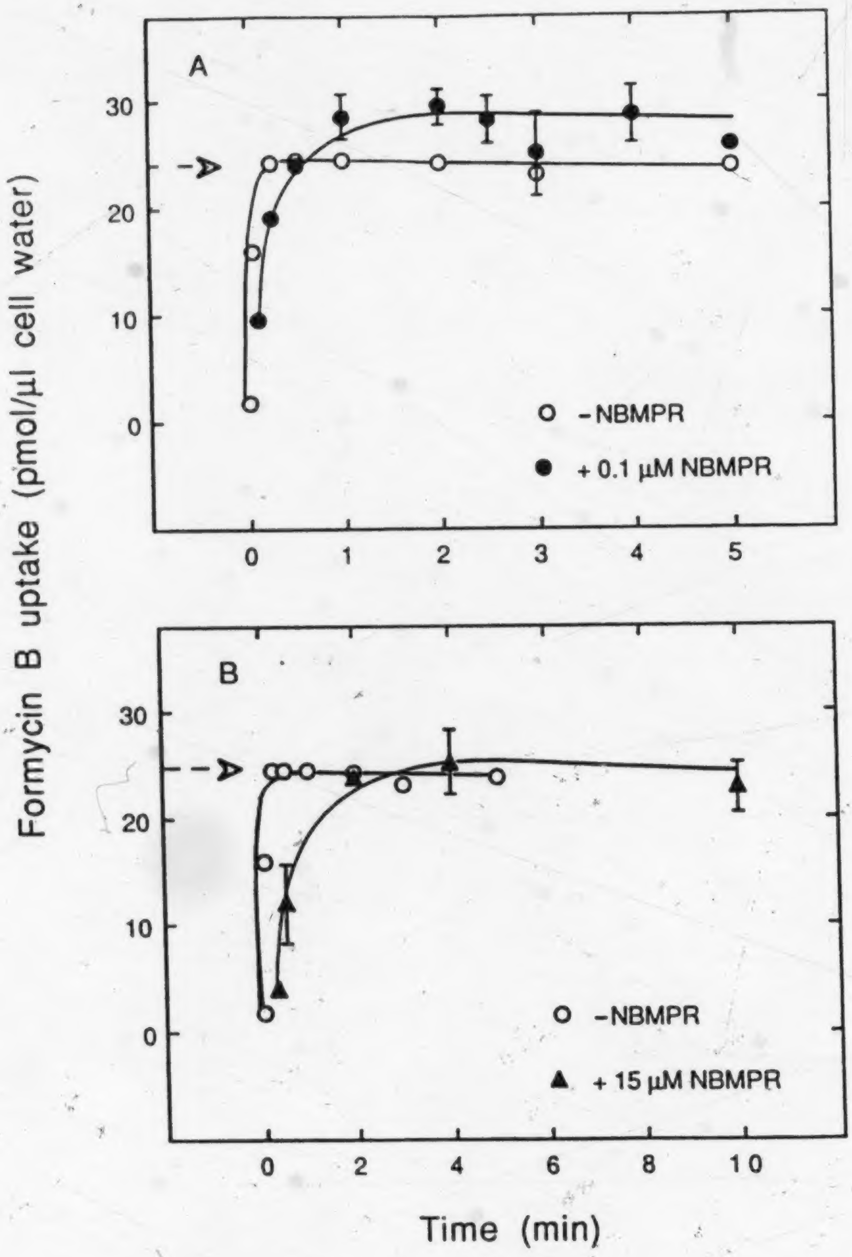
In the presence of 15 μM NBMPR, although influx of formycin B was reduced, steady state values were reached within 2-3 min, and the intracellular concentration of formycin B remained the same as the extracelluar concentration for the duration (10 min) of the experiment. The reduction in formycin B influx seen in the presence of 15 μM NBMPR suggested that the process termed "NBMPR-insensitive" could be inhibited by high concentrations of NBMPR. While formycin B influx in the presence of 15 μM NBMPR was probably the result of partial functioning of the NBMPR-insensitive process, it could also have been due to passive diffusion or an, as yet unidentified, minor transport process that was unaffected by NBMPR. The concentration of NBMPR used was more than 100-fold greater than that required to saturate the NBMPR-binding sites and was shown, in experiments presented earlier (Figure 5), to reduce transport of uridine, adenosine and thymidine by > 96%.

Although the results in Figure 16 suggested that both the NBMPR-sensitive and NBMPR-insensitive processes were equilibrative, a somewhat different result, which raised the possibility of concentrative uptake, was obtained when uptake of a higher

Figure 16

Formycin B uptake in K562/4 cells

Cellular uptake of ^{3}H-formycin B (22 µM, 20.4 µCi/ml) by actively proliferating K562/4 cells (6.5 x 10^{6}-7.5 x 10^{6} cells/ml) was measured at 37^{o}C in the presence (closed circles) or absence (open circles) of 0.1 µM NBMPR (Panel A) or 15 µM NBMPR (Panel B) by the oil-stop method as described in Figure 1. In the assays performed in the presence of NBMPR, cells were incubated at 37^{o}C in the NBMPR-containing solution for at least 10 min before the uptake measurement. The time "0" determination was conducted in the absence of NBMPR with samples individually assayed by the inhibitor-oil stop method. The dashed line with the arrow indicate the level at which the intracellular ^{3}H content was equal to the concentration of ^{3}H in the medium. Results are means of three determinations and are representative of data from one of four separate experiments. Error bars (S.D.) are shown where deviations were large enough to extend beyond the symbols.


A
B
Formycin B uptake (pmol/µl cell water)
30
20
10
0
0
1
2
3
4
5
○ -NBMPR
● + 0.1 µM NBMPR
○ -NBMPR
▲ + 15 µM NBMPR
0
2
4
6
8
10
Time (min)

concentration of formycin B was measured. In this experiment (not shown), uptake of 50 μM formycin B at 37°C was examined during a 10-min exposure in the presence and absence of 0.1 μM NBMPR. At equilibrium, the apparent intracellular concentration achieved in the absence of NBMPR was about 25-50% higher than the extracellular concentration, while the intracellular and extracellular concentrations were the same in the presence of NBMPR.

In summary, when uptake of formycin B was measured in the presence of 0.1 μM NBMPR, equilibration was seen at both 15 and 50 μM, suggesting that the NBMPR-insensitive process(es) was not capable of mediating uphill influx. When uptake was measured in the absence of NBMPR, equilibration was seen at the lower, but not at the higher, concentration of formycin B. The explanation of the apparent accumulation of formycin B at 50 μM is not clear. It is possible that at higher concentrations formycin B was slowly metabolized or bound to intracellular components as has been suggested elsewhere (Plagemann & Woffendin 1989a).

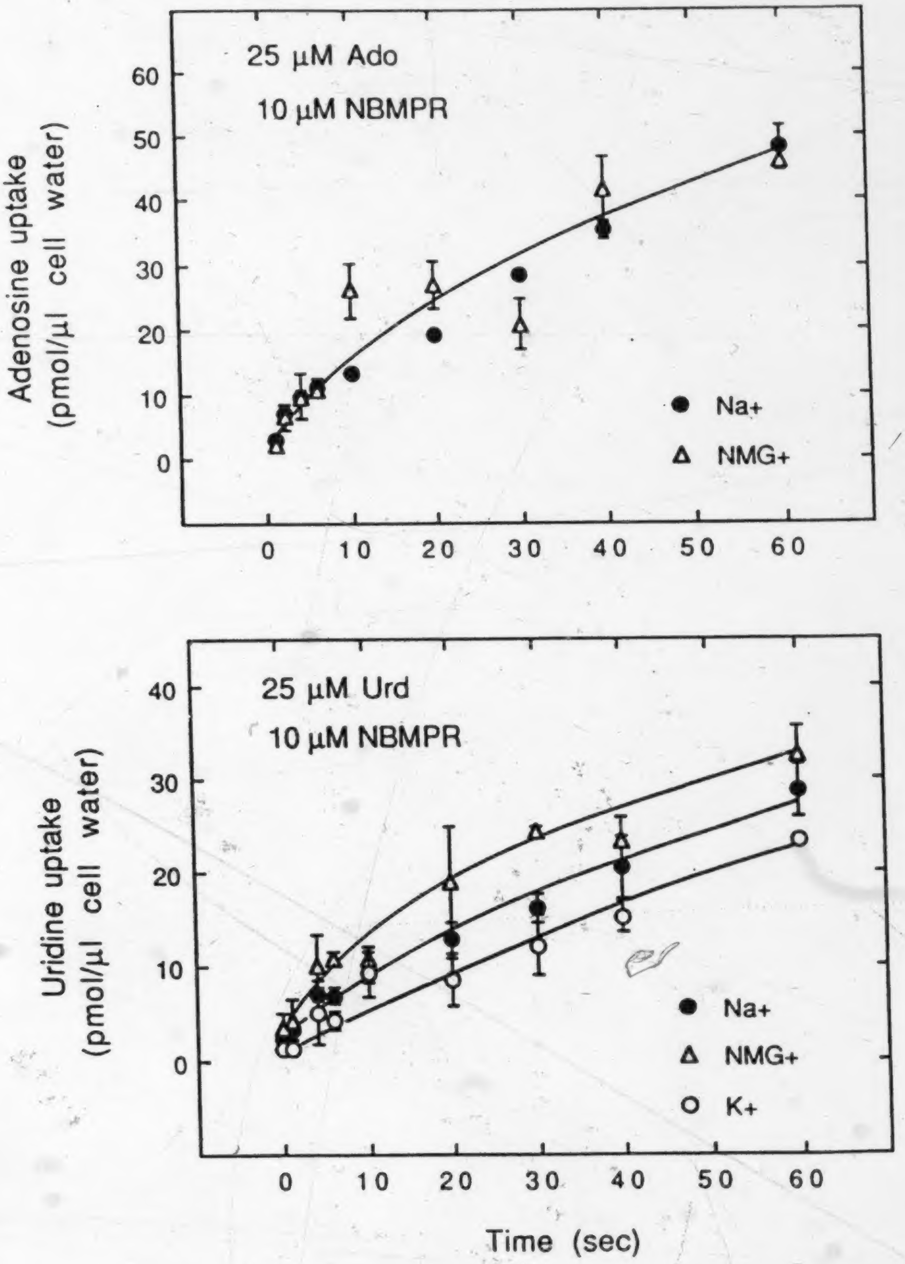
6. *Effect of Na^+ on NBMPR-insensitive transport of uridine and adenosine*

Na^+-dependent nucleoside fluxes have been identified in a variety of fresh tissue preparations (Darnowskiet al 1987, Plagemann & Woffendin 1989, Jarvis 1989, Vijayalakshmi & Belt 1988, Dagnino 1988, Lee 1989). The Na^+-linked nucleoside transport processes thus far characterized are concentrative, insensitive to inhibition by NBMPR and have narrower substrate specificities and higher affinities than the equilibrative nucleoside transport processes.

Figure 17

Effect of Na^+ on NBMPR-insensitive zero-*trans* infllux of uridine and adenosine

K562/4 cells were washed and resuspended (about 5 x 10^6 cells/ml) in isotonic phosphate-buffered Na^+, K^+, or NMG^+ transport solutions (see Chapter II, Section C.1, for composition of solutions) containing 20 μM NBMPR. Cells were first incubated at 37°C for at least 15 min in NBMPR-containing transport solution, and were then used to measure cellular uptake of ^{3}H-uridine (25 μM, 3 μCi/ml) or ^{3}H-adenosine (25 μM, 1 μCi/ml) at 37°C, in the presence of 10 μM NBMPR by the oil-stop method. Short intervals of uptake ("0" - 4 sec) were measured by the inhibitor-oil stop method and long intervals (≥ 5 sec) by the oil-stop method, as described in Figure 1. The data presented are means of two or three determinations and are from experiments conducted once (adenosine) or three times (uridine). Error bars (S.D. or M.D.) are shown where deviations were large enough to extend beyond the symbols. The initial rates of uptake were estimated as the first order coefficients of parabolas fitted to uptake data for 0-6 sec and were: for adenosine in Na^+ and NMG^+ media, respectively, 2.4 and 2.9 μM/sec; for uridine in Na^+, K^+ and NMG^+ media, respectively, 0.8, 0.7 and 1.3 μM/sec.


25 µM Ado
10 µM NBMPR
Adenosine uptake (pmol/µl cell water)
0
10
20
30
40
50
60
Na+
NMG+
0
10
20
30
40
50
60
25 µM Urd
10 µM NBMPR
Uridine uptake (pmol/µl cell water)
0
10
20
30
40
Na+
NMG+
K+
0
10
20
30
40
50
60
Time (sec)

The results obtained with formycin B suggested that the NBMPR-insensitive transport process was equilibrative, and thus likely to be independent of Na$^+$. In the experiments of Figure 17, NBMPR-insensitive uptake of adenosine and uridine was examined under conditions that should have allowed detection of a high-affinity, Na$^+$-dependent process. Uptake was assayed in the presence of 10 μM NBMPR a concentration that completely blocks the NBMPR-sensitive equilibrative process and partially blocks the NBMPR-insensitive equilibrative process. The concentration (25 μM) of adenosine and uridine used were at least one tenth of the apparent Km values estimated for the NBMPR-insensitive equilibrative process. The transport media contained Na$^+$ or were free of Na$^+$. At these permeant concentrations, the initial rates of uptake of adenosine in Na$^+$ and NMG$^+$ media were, respectively 2.4 and 2.9 μM/sec indicating that the omission of Na$^+$ had no effect on adenosine fluxes. During longer uptake intervals adenosine uptake was the same, within the range of the standard deviation, in both media. Thus, NBMPR-insensitive transport of adenosine appeared to be independent of Na$^+$ gradients.

The results obtained when the same experiment was conducted with uridine were less conclusive. The initial rates of uptake in Na$^+$, K$^+$ and NMG$^+$ (0.8, 0.7 and 1.3 μM/sec) differed slightly. These differences, which were also seen in two other experiments (not shown), indicated a small reduction in uridine transport when Na$^+$ was replaced by K$^+$ and a small increase when replaced by NMG$^+$. Since opposite effects were seen in K$^+$ and NMG$^+$ media, the differences in fluxes seemed unlikely to be due to omission of Na$^+$.

8. *Stereoselectivity of nucleoside transport in K562/4 cells*

The nucleoside transporter of human erythrocytes accepts as substrates nucleosides that differ widely in structure, although certain modifications in the ribofuranosyl moiety are not tolerated (Cass & Paterson 1972, Gati et al 1984). Recently, it was shown that the enantiomeric configuration of the ribosyl moiety is a determinant of interaction of nucleosides with the transport systems of human (Gati et al 1991) and mouse erythrocytes (Gati et al 1989), mouse leukemia L1210/AM cells (Gati et al 1989) and mouse lymphoma S49 cells (Gati et al 1991). The natural isomers, D-uridine and D-adenosine, are preferred as substrates over the L-enantiomers.

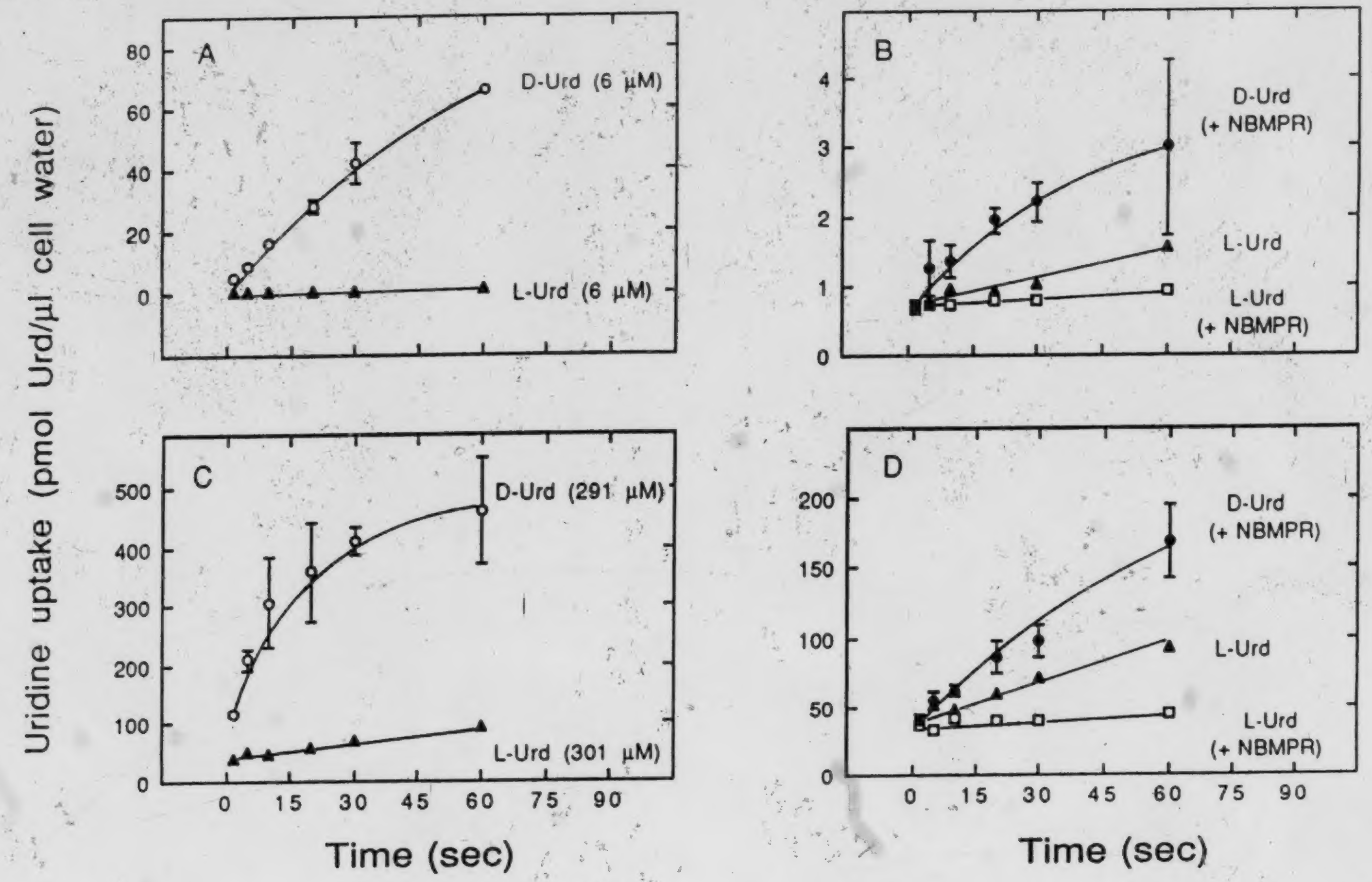
To determine if the nucleoside transport systems of K562/4 cells are stereoselective, rates of uptake of L-uridine and D-uridine were compared in assays conducted in both the presence or absence of NBMPR. In Figure 18 (Panels A and B) are shown time courses of uptake of low and high concentrations of D- and L-uridine in the absence of NBMPR. At both concentrations L-uridine permeation was much slower than that of D-uridine and visual inspection of the time courses for uptake of D- and L-uridine demonstrated that the transport was stereoselective.

The effects of 10 µM NBMPR on uptake of D- and L-uridine were also examined in the experiments of Figure 18 (Panels C and D). In the presence of 10 µM NBMPR, the inward flux (µM/sec) of 6 µM L-uridine was decreased by 89% from 0.0027 ± 0.0013 to 0.003 ± 0.001, and of 301 µM L-uridine by 68% from 0.87 to 0.28. This inhibition by NBMPR indicated (i) that entry of L-uridine was partly

Figure 18

Enantiomeric selectivity of uridine transport in K562/4 cells

Uptake of D-uridine (6 µM or 291 µM, 4.85 µCi/ml) and L-uridine (6 µM or 301 µM, 8.08 µCi/ml) was measured at 37°C in the absence (Panels A, B) or presence (Panels C, D) of 10 µM NBMPR. Uptake intervals were initiated by the addition of 100-µl portions of cell suspension (0.8 x 10^6-1.0 x 10^6 cells/ml) to equal volumes of ^{3}H-labelled nucleoside solution, and were ended by the oil-stop method as described in Figure 1. In the assays performed in the presence of NBMPR, cells were exposed to the inhibitor for at least 10 min before exposure to the ^{3}H-labelled nucleoside. The data presented here are means of duplicate determinations from one of two experiments, which gave similar results.


A
B
C
D
Uridine uptake (pmol Urd/µl cell water)
Time (sec)
Time (sec)
0
20
40
60
80
0
1
2
3
4
0
100
200
300
400
500
0
50
100
150
200
0
15
30
45
60
75
90
D-Urd (6 µM)
L-Urd (6 µM)
D-Urd (291 µM)
L-Urd (301 µM)
D-Urd
(+ NBMPR)
L-Urd
L-Urd
(+ NBMPR)

mediated and (ii) that L-uridine was accepted as a permeant by the NBMPR-sensitive transport system.

D- and L-uridine also differed in their ability to enter cells by the NBMPR-insensitive route. In the presence of 10 µM NBMPR, influx of D-uridine was greater than that of L-uridine, indicating that the NBMPR-insensitive nucleoside transporter was also stereoselective. L-Uridine uptake in the presence of 10 µM NBMPR was ≤ 1% of that for D-uridine and probably was due to passive diffusion. Since D- and L-nucleosides have identical physical properties, they would be expected to diffuse across the lipid bilayer of the plasma membrane at identical rates.

These results demonstrated that both the NBMPR-sensitive and NBMPR-insensitive systems of K562/4 cells were highly stereoselective for uridine. As well, L-uridine was a permeant, although with low activity, for the NBMPR-sensitive system.

8. *Modulation of tubercidin toxicity in K562/4 cells by NBMPR*

Permeation of a large number of nucleoside analogs across the plasma membrane is transporter mediated (Paterson et al 1981a). Strategies to ensure the activity of cytotoxic nucleosides as anticancer agents have attempted to exploit differences in nucleoside permeation characteristics between target cells and host cells to selectively direct the toxicity of the analogs to the target neoplastic cells. Studies with bone marrow cells have shown that NBMPR may protect hemopoietic stem cells, including erythroid progenitors, from the cytotoxic effects of adenosine analogs (Janowska & Cass 1987).

Entry of tubercidin (7-deazaadenosine) into cells is mediated, and in mouse lymphoma L5178Y cells, tubercidin and adenosine are competitive permeants, with similar kinetic constants for the NBMPR-sensitive transporter (Harley et al 1982). The metabolic fate of tubercidin is similar to that of adenosine with respect to phosphorylation, but, unlike adenosine, tubercidin is a poor substrate for adenosine deaminase (Suhadolnik 1979). At submicromolar levels, tubercidin has antiproliferative and cytotoxic activity against several types of cultured cells, with considerable cytotoxicity even after exposure intervals of only a few hrs (Cass et al 1982).

Resistance to tubercidin toxicity has been demonstrated in cells that lack the ability to transport nucleosides, either through mutational or pharmacological deletion (Cass et al 1981). The concentrations of tubercidin that inhibited proliferation rates of wild type mouse lymphoma S49 cells and transport-defective AE1 cells by 50% (IC_{50}) were 0.025 and 7 μM, respectively, indicating that resistance to tubercidin was conferred on the mutant cells by the loss of nucleoside transport activity. The presence of 8 μM NBMPR in the culture media conferred resistance on the wild-type cells by blocking uptake of tubercidin and the extent of resistance was similar to that seen in the transport-defective mutant cells. S49 cells express a single NBMPR-sensitive transporter and can thus be protected from tubercidin by pharmacologic blockade of transport by coadministration of NBMPR.

Modulation of tubercidin toxicity by NBMPR was examined in K562/4 cells to determine the extent to which NBMPR could protect against tubercidin toxicity in cells that coexpress NBMPR-sensitive and insensitive transport processes. Experiments were initially undertaken to determine the effective concentration of tubercidin that inhibited growth by 50% (IC_{50}). The concentration-effect relationship for tubercidin inhibition of proliferation of K562/4 cells is presented in Figure 19. Tubercidin was highly toxic with an IC_{50} value close to 0.2 µM.

The effects of NBMPR on tubercidin-induced inhibition of proliferation of K562/4 cells are shown in Figure 20. The ability of NBMPR to protect K562/4 cells from tubercidin was determined by treating K562/4 cells with graded concentrations of NBMPR alone for 30 min and then with NBMPR and various concentrations of tubercidin for 24-hr intervals. The presence of various concentrations (0.05-10 µM) of NBMPR in the culture media had only a small effect on the sensitivity of K562/4 cells to tubercidin. For 24-hr exposures, the IC_{50} value was increased 3.5-fold, from 0.2 to 0.7 µM, at the highest concentration of NBMPR and 2.5-fold, from 0.2 to 0.5 µM, at the lower concentrations of NBMPR.

Thus, NBMPR reduced tubercidin toxicity in K562/4 cells, as was indicated by the small increases in tubercidin IC_{50} values when cells were grown in the presence of various concentrations of NBMPR for 24 hrs. However, the protective effects were small, and K562/4 cells remained highly sensitive to tubercidin, despite the presence of NBMPR at concentrations that were sufficient to completely inhibit the NBMPR-sensitive transport system. Tubercidin evidently reached intracellular concentrations that were cytotoxic to K562/4 cells through uptake by the NBMPR-insensitive route.

Figure 19

Inhibition of proliferation of K562/4 cells by tubercidin Duplicate cultures (20 ml, 1 x 10^5 cells/ml) were exposed to graded concentrations of tubercidin (0.1-100 μM) over a period of 48 hrs under growth conditions similar to those described in experimental procedures for maintenance of stock cultures. Cell counting was performed at 24 and 48 hrs. The doubling time of the cell population was determined by subtracting the $\log_2$ of the initial cell population density from the $\log_2$ of the cell population density at 24 hrs. The results are expressed as % control proliferation rate defined here as the relative number of cell population doublings within 24 hrs. Each point is the mean of duplicate determinations. Data represented with different symbols (closed or open circles) are from two separate experiments.

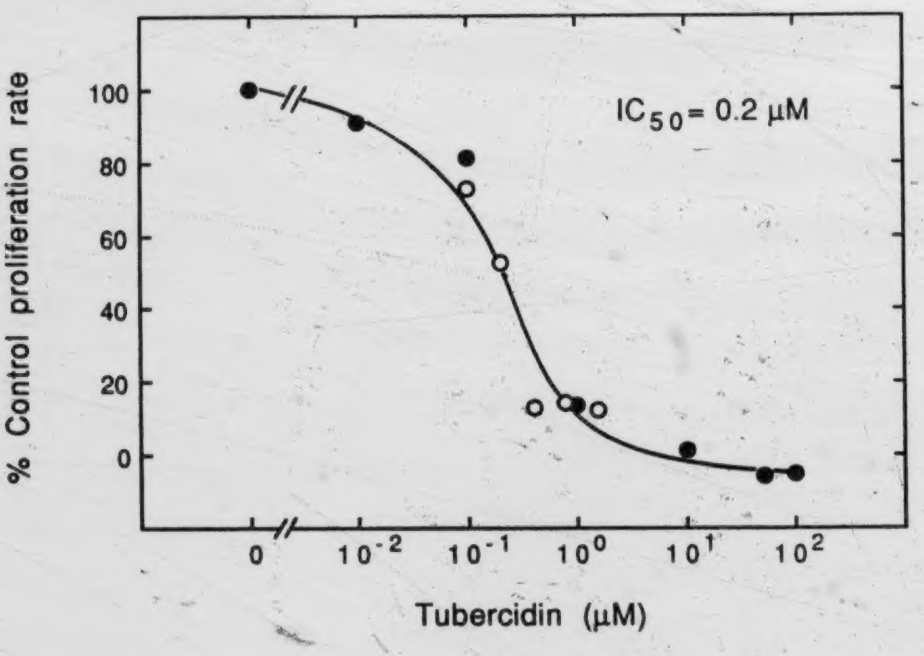
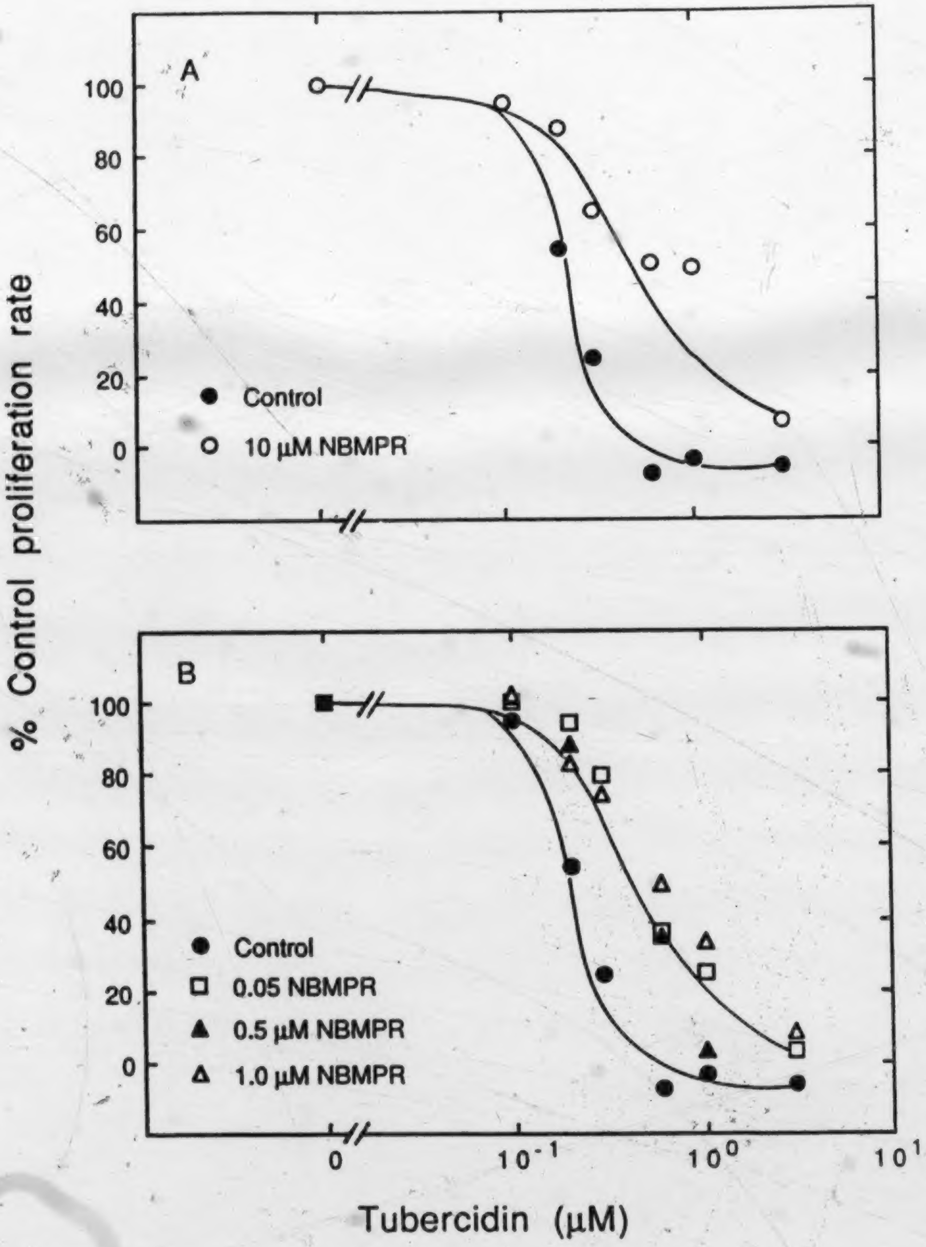

IC$_{50}$= 0.2 μM
% Control proliferation rate
100
80
60
40
20
0
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
Tubercidin (μM)

Figure 20

Effect of NBMPR on tubercidin-induced inhibition of proliferation of K562/4 cells

Cultures (2 x 10^5 cells/ml) were exposed (30-40 min at 22°C) to media without NBMPR or with graded concentrations of NBMPR (0.1-20 μM) and subsequently diluted (1 x 10^5 cells/ml) with culture media without additives or NBMPR and graded concentrations of tubercidin (0.2-6 μM). The cultures were incubated for 24 hrs under growth conditions similar to those described in Chapter II, Section B.2, for maintenance of stock cultures. Portions (1 ml) of cell suspension were withdrawn for cell counting at 24 hrs. The number of population doublings in drug treated and non-treated cultures was determined by subtracting the $\log_2$ of the initial culture density from the $\log_2$ of the culture density at 24hrs. Results are expressed as % control proliferation rate, defined here as the relative number of cell population doublings within 24hrs. Each point is the mean of duplicate determinations. For cells grown in the presence of tubercidin only, the control cultures consisted of cells incubated without additives; otherwise, control cultures were incubated in the presence of the appropriate concentration of NBMPR and the absence of tubercidin. Results are from one of two representative experiments that gave virtually identical results.


A
B
% Control proliferation rate
100
80
60
40
20
0
● Control
○ 10 μM NBMPR
● Control
□ 0.05 NBMPR
▲ 0.5 μM NBMPR
△ 1.0 μM NBMPR
0
10^{-1}
10^{0}
10^{1}
Tubercidin (μM)

C. SUMMARY

NBMPR, a potent and specific inhibitor of nucleoside transport, was used to characterize nucleoside transport in K562/4 cells by modulation of zero-*trans* influx of uridine, adenosine, thymidine and formycin B. Dilazep, another potent inhibitor of nucleoside transport, was used as a stopping reagent in "rapid sampling" methods for assaying nucleoside uptake during time intervals as short as 1 sec.

The concentration-effect relationships for inhibition by NBMPR of adenosine, uridine and thymidine zero-*trans* influx indicated that permeation of these nucleosides across the K562/4 cell membrane was mediated by at least two processes that could be distinguished on the basis of their sensitivity to NBMPR. One process was highly sensitive to inhibition by NBMPR (IC_{50} values, 0.4-1.0 nM) and the other exhibited low sensitivity to NBMPR ($IC_{50} > 5.0$ μM). The NBMPR-sensitive process comprised about 90% of the total transport activity.

K562/4 cells expressed a large number of sites ($B_{max} = 4.8 \pm 0.9 \times 10^5$ cells/ml) with high affinity for NBMPR, and the K_d value at 22°C (0.3 nM) was within the range of K_d values (0.1-1.0 nM) previously observed for erythrocytes and several other types of cultured cells. There was a good correlation between the K_d value for site-bound NBMPR at 37°C (0.5 nM) and the IC_{50} values (0.4 -1.0 nM) for inhibition by NBMPR of the major component (> 90%) of nucleoside transport activity. This correspondence of K_d and IC_{50} values suggested that the inhibition of transport was due to occupancy of the high-affinity NBMPR-binding sites.

The two nucleoside transport processes, identified on the basis of sensitivity to NBMPR, exhibited saturation kinetics for adenosine and uridine zero-*trans* influx. However, the two processes differed in their apparent affinities to bind and their capacities to transport nucleosides. The NBMPR-sensitive process had higher affinities and higher capacities than the NBMPR-insensitive process, with 5- and 3-fold higher K_m values for uridine and adenosine, respectively, and 3-fold higher V_{max} values for both nucleosides. The efficiency of permeability (π_{12}^{zt}) was much higher (10-15 fold) for the NBMPR-sensitive system, suggesting that it is the major route of uptake at low, physiologically relevant concentrations of permeant.

Both nucleoside transport processes were stereoselective, as suggested by the very low transport rates of L-uridine relative to those of D-uridine. Both processes also appeared to be equilibrative in nature. In studies of uptake of formycin B, a steady-state was reached at intracellular permeant concentrations that were the same or slightly greater than the extracellular concentrations. Uridine and adenosine fluxes mediated by the NBMPR-insensitive component were independent of Na^+ gradients.

When K562/4 cells were exposed to tubercidin in the presence of 10 μM NBMPR, a 3.5 fold increase in the IC_{50} of tubercidin was observed, and the protective effects of lower concentrations of NBMPR were even less. Thus, although transport by the NBMPR-insensitive route appeared to be insignificant for salvaging nucleosides at physiological concentrations, it may be of pharmacologic importance in that K562/4 cells could not be protected by NBMPR from cytotoxic effects of nucleoside analogs.

CHAPTER IV

IDENTIFICATION OF AN NBMPR-BINDING POLYPEPTIDE IN K562/4 CELLS AND STRUCTURAL COMPARISON WITH THE NUCLEOSIDE TRANSPORTER OF HUMAN ERYTHROCYTES

A. OVERVIEW

In human erythrocytes, polypeptides involved in NBMPR-sensitive nucleoside translocation have NBMPR-binding sites (Gati & Paterson 1989a). Reconstitution in large unilamellar vesicles of NBMPR-binding polypeptides purified from human erythrocytes resulted in reconstitution of both uridine transport and NBMPR-binding activities, establishing that both activities are properties of a single 55 kDa transmembrane polypeptide (Kwong et al 1988). Site-bound ^{3}H-NBMPR crosslinks to its binding site (Young et al 1983) upon irradiation with uv light, and because of this property, NBMPR has been used as a ligand to photolabel putative transporter polypeptides in erythrocytes from various species and a number of types of cultured cells (Gati & Paterson 1989). In some cell types, membrane polypeptides photolabelled with ^{3}H-NBMPR are comparable in size (50-56 kDa) to the human erythrocyte polypeptide, while in others the photolabelled polypeptides are larger (62-87 kDa) (Gati & Paterson 1989a). In rat erythrocytes and liver (Jarvis & Young 1986, Jarvis & Young 1987) and in cultured CCRF-CEM human T lymphoma cells (Crawford et al 1990) the difference in MW can be abolished by treatment with enzymes that remove N-linked carbohydrates. In pig erythrocytes, the molecular weight differences are maintained even after deglycosylation (Kwong et al 1986, Craik et al 1988).

An objective of this work was to identify and partially characterize NBMPR-binding polypeptides of K562/4 cells. The assumption, based on the identity of transporter polypeptides in human erythrocytes, was that NBMPR-binding polypeptides were involved in NBMPR-sensitive transport. The specific aims of the work presented in this chapter were:

(1) to determine if K562/4 cells possess NBMPR-binding polypeptides that might be components of the NBMPR-sensitive transport system, and

(2) if present, to determine by comparing structural features of the NBMPR-binding polypeptides of K562/4 cells and human erythrocytes if the NBMPR-binding polypeptides of the two cell types were similar or different.

B. RESULTS

1) *Identification of the K562/4 NBMPR-binding polypeptide by photoaffinity labelling with ^{3}H-NBMPR*

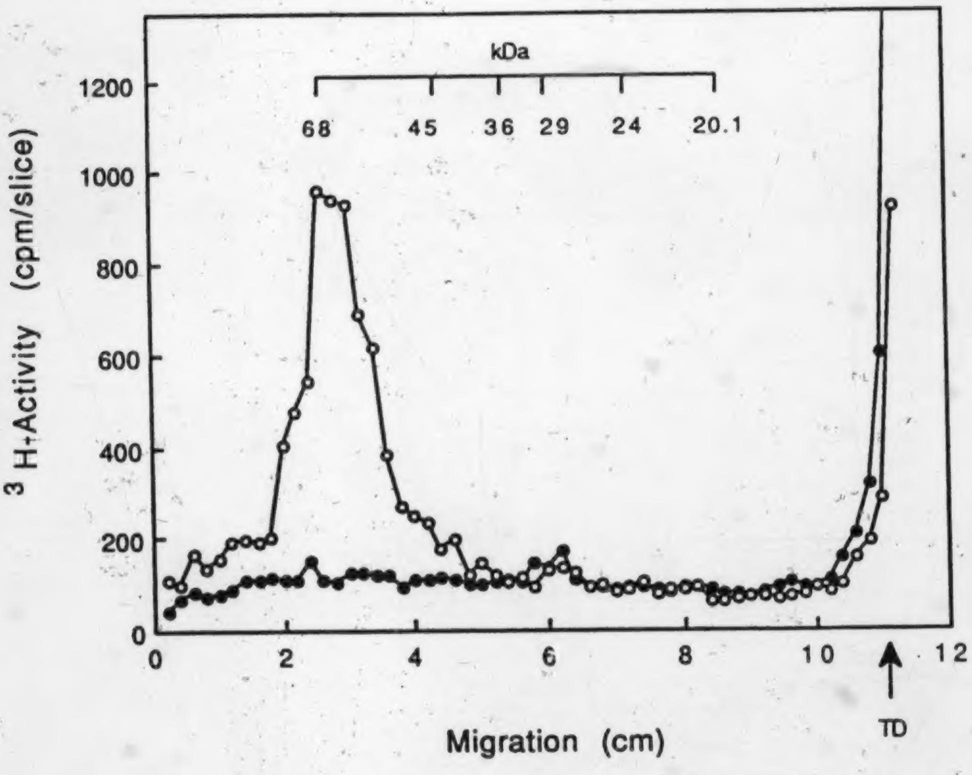
K562/4 cells expressed high-affinity binding sites for NBMPR, and occupancy of these sites correlated with inhibition of the NBMPR-sensitive nucleoside transport process (Chapter III, Section B.3). Thus, it seemed likely that K562/4 cells would possess NBMPR-binding polypeptides. To identify such polypeptides, plasma membrane preparations of K562/4 cells were photolabelled by uv activation of ^{3}H-NBMPR under conditions where the high affinity sites should be occupied.

Covalent labelling of K562/4 membrane polypeptides with ^{3}H-NBMPR was demonstrated in the experiment of Figure 21, in which

Figure 21

Analysis of ^{3}H-NBMPR-labelled K562/4 membranes by SDS-polyacrylamide gel electrophoresis

Plasma membrane-enriched fractions were prepared and photolabelled with ^{3}H-NBMPR in the absence (open circles) or presence (closed circles) of 7.5 μM non-labelled NBMPR as described in Chapter II, Sections D.1 and D.3. The photolabelled preparations were solubilized and samples (0.41 mg protein, open circles; 0.39 mg protein, closed circles) were analysed by SDS-polyacrylamide gel electrophoresis in a 12% slab gel. Migration of photolabelled polypeptides was determined by slicing each lane into 2-mm slices and estimating the ^{3}H-content in each slice by liquid scintillation counting. Migration distances of the molecular mass standards were determined by staining with Coomassie Blue and are indicated at the top of the figure. The position of the tracking dye (TD) is indicated by the arrow.


kDa
68
45
36
29
24
20.1
1200
1000
800
600
400
200
0
3 H-Activity (cpm/slice)
0
2
4
6
8
10
12
Migration (cm)
TD

membranes were photolabelled with ^{3}H-NBMPR and then subjected to SDS-polyacrylamide gel electrophoresis. The majority of the radioactivity was incorporated into a protein fraction that migrated as a broad band with an apparent molecular mass of 50-76 kDa, and a mean peak value (± S.D.) of 63 ± 3 kDa (n=10). A small fraction of radioactivity migrated faster in the region of 45 kDa, appearing as a shoulder of the major broad peak. The 45 kDa material may have been proteolyzed fragments of the major photolabelled protein. Cross-linking of ^{3}H-NBMPR to the 50-76 kDa material was abolished when photolysis was carried out in the presence of excess non-labelled NBMPR, indicating that the photolabelling of membrane polypeptides was specific. The peak of radioactivity that migrated with the tracking dye was not reduced in the presence of non-labelled NBMPR and apparently represented non-specific labelling of the lipid fraction and/or unbound ligand.

These results demonstrated the presence of a polypeptide species in K562/4 cells that migrated in SDS-polyacrylamide gels as a broad band with an average apparent molecular mass of 63 ± 3 kDa. This polypeptide may be the NBMPR-sensitive nucleoside transporter of K562/4 cells.

2) *Comparison of the electrophoretic migration profiles of the ^{3}H-NBMPR-labelled polypeptides of K562/4 cells and human erythrocytes*

The results of the photolabelling experiments described in the preceding section suggested that the apparent molecular mass of the ^{3}H-NBMPR-labelled polypeptides of K562/4 cells was higher than that of the nucleoside transporter polypeptide of human erythrocytes. To rule out the

Figure 22

Analysis of ^{3}H-NBMPR photolabelled membranes from K562/4 cells and human erythrocytes by SDS-polyacrylamide gel electrophoresis

Plasma membrane-enriched fractions from K562/4 cells and protein-depleted membranes from human erythrocytes were prepared and separately photolabelled as described in Chapter II, Sections D1 and D3. Samples of photolabelled membranes from K562/4 cells (246 µg protein, 7500 cpm), erythrocytes (60 µg protein, 7740 cpm) and molecular mass standards were then subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions in separate lanes of the same 15% slab gel. The migration distances of ^{3}H-labelled polypeptides and molecular mass standards were determined as described in Figure 22. The position of the tracking dye (TD) is indicated by the arrow.

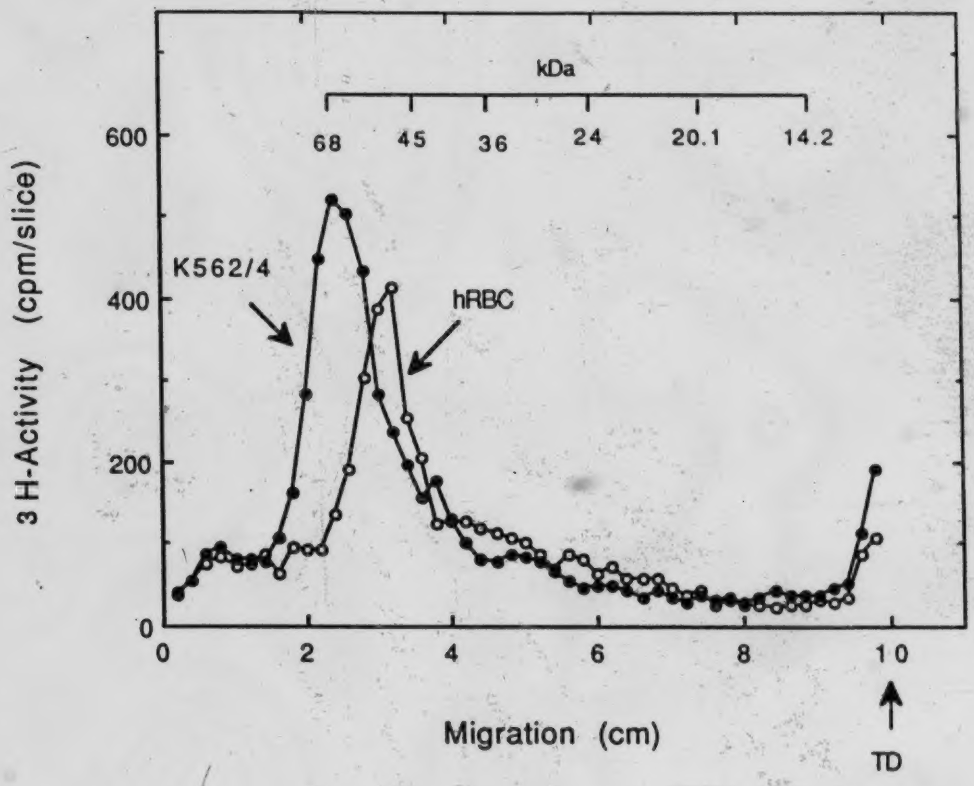

kDa
68
45
36
24
20.1
14.2
600
400
200
0
K562/4
hRBC
3 H-Activity (cpm/slice)
0
2
4
6
8
10
Migration (cm)
TD

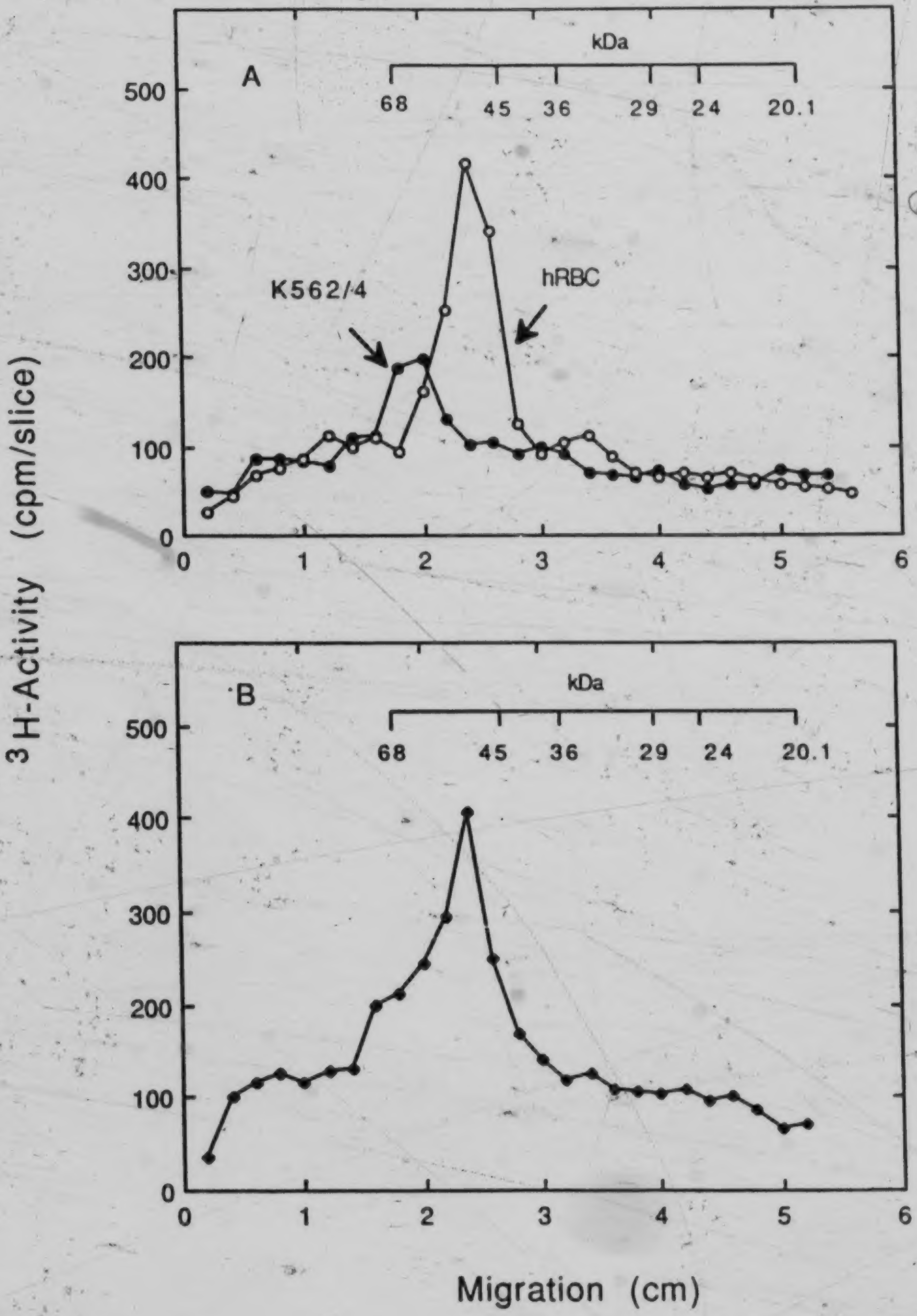
Figure 23

Analysis of a mixture of photolabelled NBMPR-binding polypeptides from human erythrocyte and K562/4 membranes by SDS-polyacrylamide gel electrophoresis

Plasma membrane-enriched fractions from K562/4 cells and protein-depleted membranes from human erythrocytes were prepared and separately photolabelled as described in Chapter II, Sections D1 and D3. The migration distances of ^{3}H-labelled polypeptides and molecular mass standards were determined as described in Figure 22.

Panel A. Samples of photolabelled membranes from K562/4 cells (69 μg protein, 14,500 cpm), human erythrocytes (80 μg protein, 10,320 cpm) and molecular mass standards were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions in separate lanes of the same 12% mini gel.

Panel B. A mixture of the photolabelled membranes from K562/4 cells (69 μg protein, 14,500 cpm) and human erythrocytes (80 μg protein, 10,320 cpm) was applied to an adjacent lane of the same 12% mini gel described in panel A.


A
kDa
68
45
36
29
24
20.1
K562/4
hRBC
B
3 H-Activity (cpm/slice)
Migration (cm)
0
100
200
300
400
500
0
1
2
3
4
5
6

possibility that these differences in molecular mass were simply due to different experimental conditions, photolabelled membranes from K562/4 cells and human erythrocytes were analyzed in the same SDS-polyacrylamide gels.

In the experiment of Figure 22 the ^{3}H-NBMPR-labelled polypeptides of K562/4 cells migrated more slowly than the ^{3}H-NBMPR-labelled polypeptides of human erythrocytes, with a peak molecular mass of 64 kDa. The human erythrocyte polypeptides migrated with a peak molecular mass of 53 kDa, which was close to the values (55 kDa) reported in the literature (Kwong et al 1989).

In the experiment of Figure 23, K562/4 and human erythrocyte membranes were separately photolabelled and then subjected to electrophoretic analysis either in separate lanes (Panel A) or together in a single lane (Panel B) of the same gel. The radioactive material present in the sample consisting of both K562/4 and human erythrocyte membranes migrated as a broad band (peak values 50-55 kDa), with a distinct shoulder (64-68 kDa). The peak corresponded in its position to the photolabelled polypeptides seen in the analysis of erythrocyte membranes and the shoulder to the photolabelled polypeptides of K562/4 membranes.

Thus, the ^{3}H-NBMPR-labelled polypeptides of K562/4 cells migrated during SDS-polyacrylamide gel electrophoresis as though they were larger than the ^{3}H-NBMPR-labelled polypeptides of human erythrocytes. The difference in apparent molecular mass as resolved in the electrophoretograms was 10-12 kDa.

3) *Deglycosylation of NBMPR-binding polypeptides of K562/4 cells and human erytrocytes*

NBMPR-binding polypeptides from several cell types exhibit molecular masses that are larger than that of the nucleoside transport protein of human erythrocytes. There are at least two examples (CEM cells, rat erythrocytes) (Crawford et al 1990, Jarvis & Young 1986) in which deglycosylation of membrane proteins reduces the apparent molecular mass of ^{3}H-NBMPR-labelled polypeptides to values similar to that of the deglycosylated protein of human erythrocytes (Kwong et al 1986).

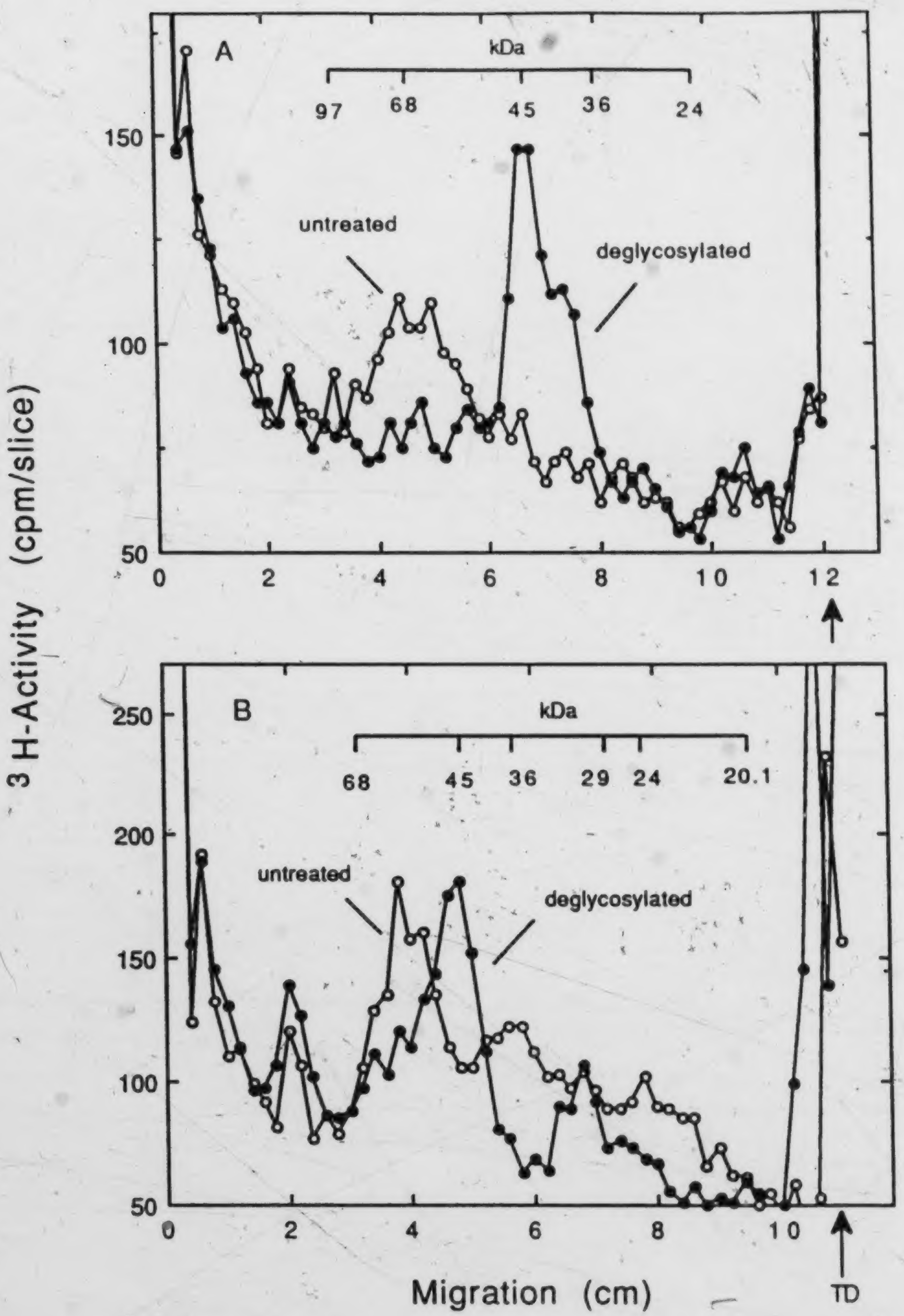
To determine if the larger molecular mass of the NBMPR-binding polypeptides of K562/4 cells was due to carbohydrates, ^{3}H-NBMPR labelled membranes of K562/4 cells and human erythrocytes were treated with N-glycosidase F, which hydrolyses asparagine-linked oligosaccharides, and then analysed by SDS-polyacrylamide gel electrophoresis (Figure 24). Treatment of K562/4 membranes with N-glycosidase F (Panel A) converted the ^{3}H-labelled polypeptides from material that migrated as a broad band (peak value, 67 kDa) to material that migrated more rapidly as a sharper band (peak value, 43 kDa). Treatment of erythrocyte membranes with N-glycosidase F (Panel B) reduced the apparent molecular mass of the ^{3}H-labelled polypeptides from 55 kDa to 45 kDa.

Thus, the deglycosylated polypeptides migrated as sharper bands with peak values of 42 ± 2 kDa (mean ± S.D. of 3 experiments) and 44 ± 2 kDa (mean ± M.D. of 2 experiments) for K562/4 cells and human erythrocytes, respectively. These results suggested that the

Figure 24

Effect of N-glycosidase F on the electrophoretic mobility of ^{3}H-NBMPR-labelled polypeptides from K562/4 cells and human erythrocytes

Plasma membrane-enriched fractions from K562/4 cells and protein-depleted membranes from human erythrocytes were prepared and separately photolabelled as described in Chapter II, Section D.1 and D.3. K562/4 membranes (103 μg protein) were incubated as described in Chapter II, Section D.5, with (closed circles) or without (open circles) N-glycosidase F (0.190 U/20μg of protein) for 105 min at 37°C (**Panel A**). Erythrocyte membranes (100 μg protein) were incubated with (closed circles) or without (open circles) N-glycosidase F (0.135 U/20 μg protein) for 1 h at 37°C (**Panel B**). After digestion with N-glycosidase F, samples were analysed together with molecular mass standards by SDS-polyacrylamide gel electrophoresis under reducing conditions in a 12% slab gel as described in Figure 22. The position of the tracking dye (TD) is indicated by the arrow.


A
kDa
97
68
45
36
24
untreated
deglycosylated
150
100
50
0
2
4
6
8
10
12
B
kDa
68
45
36
29
24
20.1
250
200
150
100
50
untreated
deglycosylated
0
2
4
6
8
10
TD
3 H-Activity (cpm/slice)
Migration (cm)

difference in apparent molecular mass between the NBMPR-binding polypeptides of K562/4 cells and human erythrocytes were mainly due to differences in glycosylation.

Although proteolytic inhibitors were included in the reaction mixture, extensive proteolysis was observed during the incubations at 37°C. Proteolysis was more pronounced in samples of erythrocyte membranes than in samples of K562/4 membranes. Another limitation was the low recovery of radioactivity. Deglycosylation of membrane proteins seemed to promote aggregation of the ^{3}H-labelled material, which was evident in the extensive accumulation of radioactivity in the begining of the separating gels.

4) *Peptide mapping of the NBMPR-binding polypeptides of K562/4 cells and human erythrocytes*

The ^{3}H-NBMPR-binding polypeptides of K562/4 cells and human erythrocytes appeared to be of similar sizes after deglycosylation. The next question was whether the two proteins had the same, or related, primary structures. To address this question, experiments were undertaken to establish conditions for peptide mapping of the NBMPR-binding polypeptides of K562/4 cells and human erythrocytes.

Peptide map analysis by limited proteolysis and SDS-polyacrylamide gel electrophoresis as described by Cleveland (Cleveland et al 1976) can be used to establish the relatedness of polypeptides at the level of primary structure. With this method, the migration pattern of peptide fragments generated by digestion of

Figure 25

Effect of V8 protease on the electrophoretic mobility of ^{3}H-NBMPR-labelled polypeptides of K562/4 cells and human erythrocytes

Plasma membrane-enriched fractions from K562/4 cells and protein-depleted membranes from human erythrocytes were prepared and separately photolabelled as described in Chapter II, Sections D1 and D3. K562/4 and erythrocyte membranes were solubilized in electrophoresis sample buffer and loaded on a 1.5-mm thick slab gel consisting of 4% (5 cm) stacking gel and 15% (10 cm) separating gel. V8 protease (1μg enzyme/100μg protein) was added to the appropriate wells and digestion was allowed to take place as proteins moved slowly in the stacking gel (140 min, at RT). A 40 mA current was applied and raised to 60 mA when samples reached the interface between the stacking and separating gels The ^{3}H-profile shown was determined by slicing the gel immediately after electrophoresis and determining the ^{3}H-content of each slice.

Panel A. K562/4 membranes were undigested (open circles: 246 μg protein, 7500 cpm) and digested (closed circles: 328 μg protein, 10,000 cpm) with 3.5 μg of V8 protease.

Panel B. Human erythrocyte membranes were undigested (open circles: 60 μg protein, 7740 cpm) and digested (closed circles: 80 μg protein, 10320 cpm) with 0.8 μg of V8 protease.

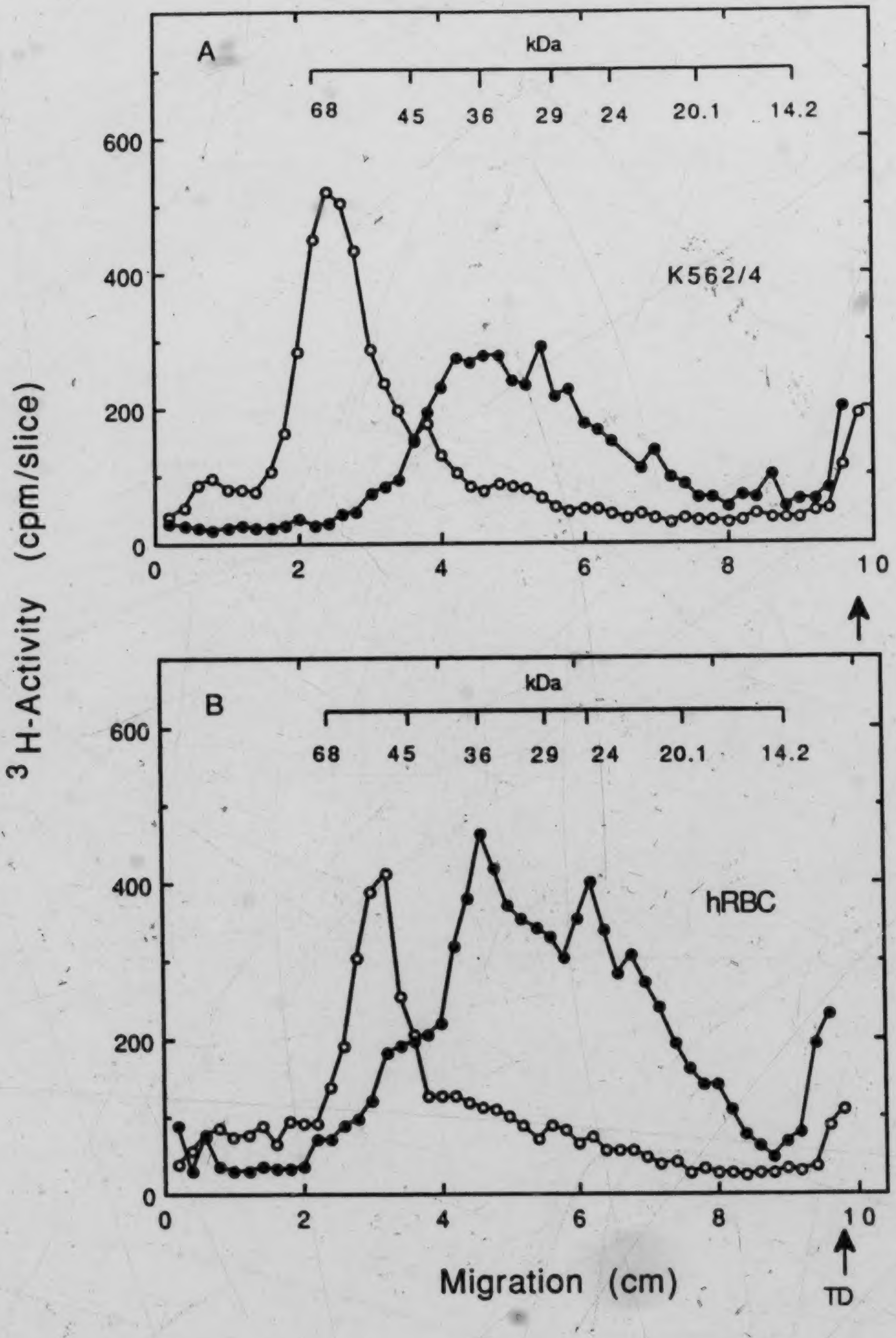

A
kDa
68
45
36
29
24
20.1
14.2
K562/4
B
hRBC
3 H-Activity (cpm/slice)
600
400
200
0
0
2
4
6
8
10
Migration (cm)
TD

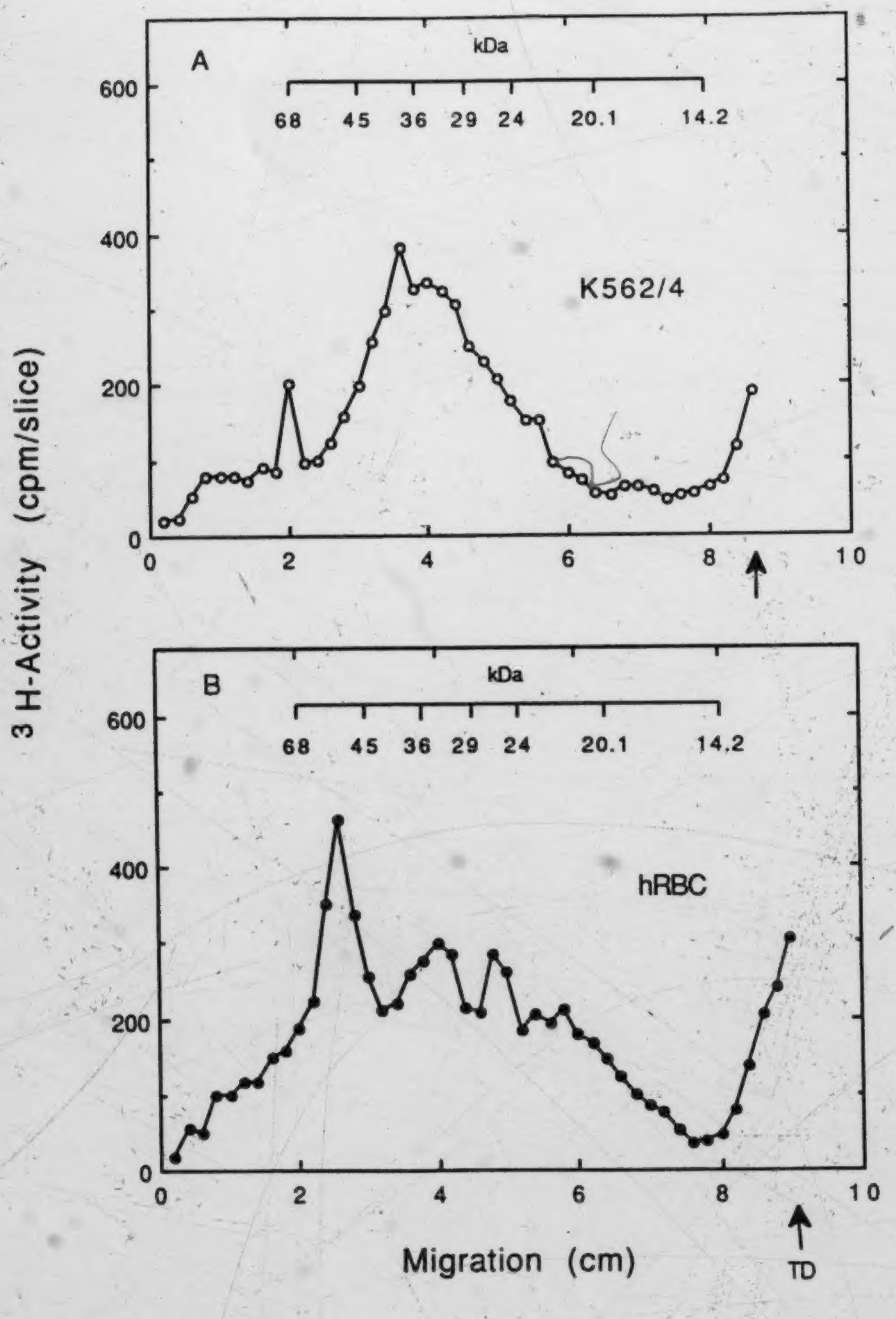
Figure 26

Effect of V8 protease on the electrophoretic mobility of ^{3}H-NBMPR-labelled polypeptides of K562/4 cells and human erythrocytes

Proteolytic digestion of and K562/4 and erythrocyte photolabelled membranes was conducted as described in Figure 26 with these differences: (i) digestion was carried out in a 2.5-cm 4% stacking gel and the current was stopped for 15 min when proteins reached the stacking-separating gel interface, (ii) proteolytic fragments were analyzed in a 12% gel (10 cm long, 3 mm thick) and (iii) different quantities of enzyme were used for proteolysis.

Panel A. K562/4 membranes (340 μg protein, 9860 cpm) were digested with 2.2 μg of V8 protease (1 μg enzyme/150 μg protein).

Panel B. Human erythrocyte membranes (125 μg protein, 9250 cpm) were digested with 2 μg of V8 protease (1 μg enzyme/63 μg protein).


A
kDa
68
45
36
29
24
20.1
14.2
600
400
200
0
K562/4
0
2
4
6
8
10
B
kDa
68
45
36
29
24
20.1
14.2
600
400
200
0
hRBC
0
2
4
6
8
10
3 H-Activity (cpm/slice)
Migration (cm)
TD

protein in its denatured state is characteristic of the protein substrate and the specific protease used. Possible relationships between polypeptides are determined by comparing the patterns of peptide migration on the gel, and, if two peptide profiles are dissimilar, the original polypeptides are not closely related.

Staphylococcus aureus V_8 protease is a serine protease which specifically cleaves peptide bonds C-terminally at glutamic and aspartic acid residues (Houmard & Drapeau 1972, Drapeau 1978). The enzyme retains 77% of its activity in the presence of 0.1% SDS, a property that, along with its specificity, has made it one of the preferred enzymes for peptide mapping by the Cleveland method.

Figures 25 and 26 show the electrophoretic patterns of ^{3}H-labelled fragments generated by limited proteolysis with *Staphylococcus aureus* V_8 protease of ^{3}H-NBMPR labelled polypeptides from K562/4 cells and human erythrocytes. In Figure 26, which shows profiles for untreated material, it is evident that non-digested photolabelled polypeptides migrated as single broad bands. Thus, the membranes used for these analyses had not undergone endogenous proteolysis.

In the experiment of Figure 25 A, treatment of K562/4 membranes with V8 protease (1 μg of enzyme/100 μg of protein) resulted in almost complete disappearence of the original ^{3}H-labelled material, which migrated as a well defined band (peak value 64 kDa), and the appearence of smaller fragments, which migrated diffusely in the region of 24-45 kDa. In the experiments of Figure 26A, when less enzyme was used (1 μg of enzyme/150 μg of protein), the original ^{3}H-labelled material was nearly completely digested to fragments that labelled material was nearly completely digested to fragments that migrated in the region of 22-48 kDa. When more enzyme was used (1 μg of enzyme/20 μg of protein), the 22-48 kDa fragments remained and were not further modified (data not shown). The diffuse migration pattern suggested that the ^{3}H-labelled fragments produced by V8 protease digestion were heterogeneously glycosylated.

Different results were obtained when erythrocyte membranes were digested with V8 protease. In the experiment of Figure 25B, the ^{3}H-labelled fragments migrated in two regions, of 29-42 kDa (peak value, 36 kDa) and 22-27 kDa (peak value, 26 kDa). In the experiment of Figure 26B, when more enzyme was used (1 μg enzyme/63 μg protein), the original ^{3}H-labelled material was not completely digested and radioactivity was found in the original 43-65 kDa band (peak value, 54 kDa) and three smaller bands of 29-40 kDa (peak value, 35 kDa), 24-28 kDa (peak value, 25 kDa) and 19-24 kDa (peak value, 22 kDa).

Thus, the NBMPR-binding polypeptides of K562/4 cells and human erythrocytes exhibited different patterns of fragmentation when they were subjected to limited proteolysis in their denatured state by *Staphylococcus aureus* V_8 protease. Digestion of K562/4 membranes produced ^{3}H-labelled material that migrated as a broad band in the region of 22-48 kDa. Digestion of human erythrocyte membranes produced ^{3}H-labelled material that migrated as 3 peaks, at 35 kDa, 25 and 22 kDa. These differences in fragmentation patterns may be due to differences in glycosylation. However it is possible that other differences in the primary structure of the K562/4 and erythrocyte proteins may exist.

C. SUMMARY

NBMPR-binding polypeptides were identified in plasma membrane fractions from K562/4 cells by irradiation with uv light in the presence of site bound ^{3}H-NBMPR. A polypeptide species that was cross-linked with the ^{3}H-ligand migrated in electrophoretograms as a broad band of 50-76 kDa (peak value 63 kDa) and thus appeared to be larger than the nucleoside transport protein of human erythrocytes. When analysed in the same SDS-polyacrylamide gels, ^{3}H-NBMPR-labelled polypeptides of K562/4 cells and human erythrocytes migrated as broad bands, but with different electrophoretic mobilities, that differed in their peak values for apparent molecular mass by 10-12 kDa. This difference in apparent molecular mass was completely abolished by deglycosylation of membrane proteins with N-glycosidase F and thus appeared to be due to different amounts of N-linked carbohydrates. The K562/4 and erythrocyte proteins exhibited different fragmentation patterns when subjected to peptide mapping by limited digestion with *Staphylococcus aureus* V8 protease. The ^{3}H-NBMPR-labelled fragments of K562/4 cells migrated as a broad band (22-58 kDa), whereas those of erythrocytes migrated as sharper bands (peak values 35, 25 and 22 kDa).

CHAPTER V

CHANGES IN NUCLEOSIDE TRANSPORT ACTIVITY IN K562/4 CELLS UPON HEMIN TREATMENT

A. OVERVIEW

Membrane transport changes during differentiation.

i) *Erythroid cells*

Within 24-48 hrs of being released into the circulation, reticulocytes lose intracellular organelles and functions that distinguish them from erythrocytes (Gronowicz et al 1984). During maturation reticulocytes undergo substantial changes in the composition of the plasma membrane, including loss or reduction of nucleoside transport (Tucker & Young 1980, Jarvis & Young 1982), glucose transport (Zeidler et al 1982), Na^+-independent amino acid transport and Na^+-glycine cotransport (Johnstone et al 1987, Blostein & Grafova 1987). Decreases have also been observed in Na^+/K^+ ATPase (Blostein et al 1983), acetylcholinesterase (Conscience et al 1977) and transferrin binding activities (Frazier et al 1982, Pan et al 1983). The mechanism(s) responsible for the changes in membrane transport processes during reticulocyte maturation is not clear. It has been suggested that transport components may be lost by membrane recycling and exocytosis, as well as by a pathway involving targeting to and degradation in intracellular organelles (Blostein & Grafova 1987). Others have suggested that ATP-dependent release of membrane vesicles may be responsible for the loss of specific membrane functions during *in vitro* maturation of reticulocytes to erythrocytes (Johnstone et al 1987).

A decrease in nutrient transport activity was observed (Mager & Bernstein 1978, Gazzit 1979, Guidotti et al 1978) during erythroid differentiation of Friend mouse erythroleukemia cells. In a more recent study, Gordon and Rubin (1991) showed that during treatment of Friend cells with dimethylsulfoxide, fluxes for monosacharides, leucine, aminoisobutyric acid and lysine increased, while those for uridine remained unchanged. The difference in this study, relative to earlier studies, was that changes in cell volume during maturation were taken into consideration.

Differences in membrane transport have also been observed between differentiated and immature non-erythroid cells. Freshly isolated, immature cells from leukemic patients showed differences in membrane transport activity when compared with their mature counterparts. AraC influx and the maximum binding of NBMPR for myeloblasts and lymphoblasts from patients with acute myeloid, acute lymphoid or undifferentiated leukemias were higher (2-6 fold) than for the normal mature cells (Wiley et al 1982).

In HL60 cultured promyelocytic leukemia cells, Na^+/H^+ exchange activity decreases during N,N'-dimethylformamide-induced aquisition of the granulocytic phenotype (Chen et al 1986). In addition, induction of HL-60 cells to granulocytic differentiation by treatment with dimethyl sulfoxide results in an increase in Na^+-dependent uridine transport and a decrease in facilitated diffusion that correlates with a decrease in NBMPR-binding sites (Costa-Casnellie et al 1988, Lee 1990). A recent study demonstrated that Na^+

dependent nucleoside transport is expressed in confluent cultures of IEC-6 mouse intestinal epithelial cells, which are believed to have crypt cell origin. The increased activity of the Na$^+$-dependent nucleoside transport system in IEC-6 cells was suggested to be a result of cellular differentiation (Jakobs et al 1991) in responce to factors (e.g., insulin, epidermal growth factor and hydrocortisone) present in the culture fluids.

Hemin[3] as an erythroid differentiation inducing agent.

i) *Normal erythroid cells*

Heme[4], a structural component of hemoglobin, controls the efficiency of translation of globin mRNA, and thus also hemoglobinization of erythrocytes, by affecting the activity of initiation factors for protein synthesis (Ochoa & De Haro 1979, Maitra et al 1982). It is known that a cAMP-dependent protein kinase, the activity of which is regulated by heme, is involved in the control of the translation initiation factor, eIF2 (Maitra et al 1982). In reticulocytes, the synthesis of globin, together with that of other less abundant proteins, depends on the presence of heme (London et al 1976). For example, 20-40 μM hemin is required to maintain protein synthesis for prolonged periods in lysates of rabbit reticulocytes (Zucker & Shulman 1978).

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[3] Hemin (Chlorohemin): 1,3,5,8-tetramethyl-2,4-divinylporphine-6,7-dipropionic acid ferrichloride.

[4] Heme: 1,3,5,8-Tetramethyl-2,4-divinylporphine-6,7-dipropionic acid ferrous complex

Heme may also be involved in maturation of erythroid progenitor cells. Addition of 100-200 μM hemin to primary two-day cultures of mouse bone marrow cells enhanced growth of erythroid colonies, even in the absence of erythropoietin (Porter et al 1979). In cultures of immature erythroid cells derived from the bone marrow of anemic rabbits, exogenous hemin (10-100 μM) resulted in a concentration-dependent reduction in DNA synthesis and proliferative capacity of cells, with an increase in the proportion of cells synthesizing hemoglobin. However, these results are hard to interpret because hemin also causes a non-specific decrease in DNA synthesis in non-erythroid cells (Bonanou-Tsedaki 1981). Nonetheless, an increase in intracellular heme, by whatever mechanism, leads to enhanced cell maturation by accelerating the rate of hemoglobin synthesis. Heme released in plasma as a result of severe hemolysis may play a physiological role in the replenishment of circulating blood cells by rapidly increasing the rate of erythroid cell maturation in the bone marrow (Bonanou-Tsedaki et al 1981).

ii) *K562 cells*

Treatment of K562 cells with hemin induces, or enhances, expression of erythroid features that are more characteristic of early embryonic or fetal erythroid cells than of adult erythoid cells. Expression of i surface antigen (a carbohydrate antigen characteristic of fetal erythrocytes), embryonic isoenzymes of lactate dehydrogenase, embryonic and fetal globin, and embryonic and fetal globin mRNA is enhanced by exposure of K562 cells to hemin (Benz et al 1980, Rutherford et al 1979, Charnane & Maniatis 1983, Dean 1983) and it has been suggested that hemoglobin induction is mediated at the transcriptional level (Dean et al 1983). Hemin treatment also causes changes in the activities of enzymes known to be associated with erythroid differentiation. For example, heme biosynthetic enzyme activities rise and catabolic activities fall (Hoffman et al 1980). Another effect that appears to be specifically related to hemin-induced erythroid differentiation in K562 cells is reduction in the bcr/c-abl protein in the absence of general reduction in protein synthesis (Richardson et al 1987). The level of the bcr/c-abl tyrosine kinase activity and the phosphotyrosine content of its substrate proteins are also reduced during hemin-induced differentiation.

Although hemin treatment results in expression of erythroid features, it does not lead to irreversible erythroid differentiation or commitment of K562 cells. Removal of hemin from culture media after a 14-day exposure resulted in reduction of hemoglobin expression to levels found in cells growing in the absence of hemin. (Dean et al 1981).

Objectives

The membrane-associated changes that occur during the course of maturation of reticulocytes and other cells raised the possibility that induction of differentiation along the erythroid lineage might also cause changes in the transport of nucleosides in K562 cells. The aim of the work presented in this chapter was to establish conditions for studies of nucleoside transport with K562 cells induced to erythroid differentiation. K562/4 cells were treated with hemin and changes in the nucleoside transport capacity of hemin-treated K562/4 cells were examined by assessing (i) NBMPR-binding activity and (ii) zero-*trans* influx of adenosine. Erythroid differentiation in hemin-treated cultures was assessed by monitoring hemoglobin accumulation in hemoglobin-producing cells.

B. RESULTS

1) *Effect of hemin treatment on proliferation and hemoglobin expression in K562/4 cells*

Earlier studies (Sutherland 1984) indicated that treatment of K562/4 cells with 25 µM hemin induced production of hemoglobin, as indicated by an increase in the proportion of benzidine-positive cells, during the course of 4-day exposures. Increased expression of early myeloid markers and glycophorin and a decrease in cytoplasmic glycogen were also observed in hemin-treated K562/4 cells. Since the concentration of cytoplasmic glycogen decreases as erythroid differentiation proceeds (Breton-Goriuset al 1981), this change, together with the others, were interpreted (Sutherland 1984) as a return to more normal erythroid metabolism or of erythroid differentiation.

In the experiments of Figure 27, cultures of actively proliferating K562/4 cells were exposed to 50 µM hemin for up to 4 days. The growth rates of untreated (control) and hemin-treated K562/4 cells were only slightly different (Figure 27A), and, after 4 days of exposure to hemin, viability was reduced, relative to that of control cells, not more than 26% (Figure 27B). In hemin-treated cultures, the number of benzidine-positive cells increased to a maximum of 80-85% after 2 days (Figure 27C), whereas there were

Figure 27

Effect of hemin treatment on proliferation, viability and hemoglobin expression in K562/4 cells

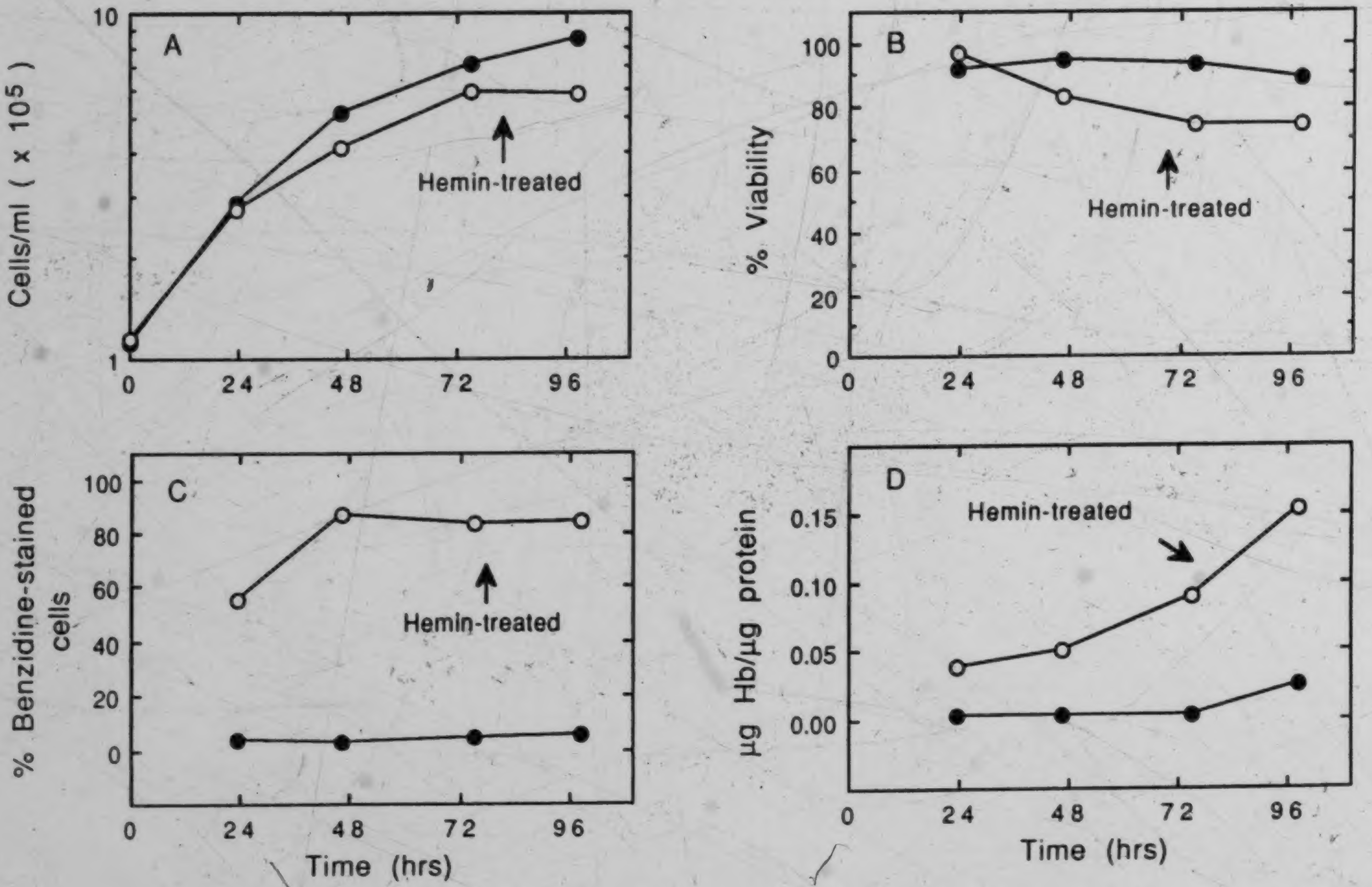
Cultures (100 ml) of K562/4 cells were initiated at 1 x 10^5 cells/ml and grown in the presence (open circles) or absence (closed circles) of 50 µM hemin for 4 days. Samples were withdrawn from the cultures at 24-hr intervals for assessment of population density, viability, % of cells producing detectable amounts of hemoglobin and levels of hemoglobin accumulation as described in Chapter II, Sections B2,4,6. Results are from a single representative experiment, and each point represents one determination.

[A] : Growth curves. Cell concentrations were determined by electronic particle counting of 1-ml samples

[B] Cell viability. Viable cells were identified under a light microscope by their ability to exclude trypan blue using a minimum of 100 cells.

[C] Proportion of cells producing hemoglobin. The percentage of positive cells in the benzidine oxidation test was determined using a minimum of 200 cells.

[D] Hemoglobin accumulation. The hemoglobin (Hb) and protein contents of cell lysates were determined by the benzidine and micro Lowry assays.


A
Cells/ml (x 10^5)
10
1
0
24
48
72
96
Hemin-treated
B
% Viability
100
80
60
40
20
0
0
24
48
72
96
Hemin-treated
C
% Benzidine-stained cells
100
80
60
40
20
0
0
24
48
72
96
Time (hrs)
Hemin-treated
D
μg Hb/μg protein
0.15
0.10
0.05
0.00
0
24
48
72
96
Time (hrs)
Hemin-treated

only occasional benzidine-positive cells in the untreated cultures. The benzidine-positive cells varied in staining intensity.

The production of hemoglobin in hemin-treated cultures increased with time of exposure to hemin, and the greatest increase was seen after 3 days of exposure (Figure 27D). The hemoglobin content of untreated cultures remained constant for 3 days and then increased slightly on the fourth day, when the culture population density was 8.4 x 10^5 cells/ml. K562 cells grown to high cell population densities in the absence of hemin have been shown before (Trentesaux et al 1989) to exhibit small increases in hemoglobin production.

The results presented in Figure 27 demonstrate that K562/4 cells responded to continuous treatment with 50 µM hemin with a substantial increase in hemoglobin production and, relative to control cells, with only small changes in proliferative ability and viability. Since enhancement of hemoglobin production in treated cells indicated induction of a differentiation response, cells were treated with 50 µM hemin in subsequent studies.

2) *Effect of hemin treatment on binding of NBMPR to K562/4 cells*

In erythrocytes that possess NBMPR-sensitive nucleoside transport systems, the number of NBMPR-binding sites has been correlated with nucleoside transport capacity (Cass et al 1974). As an initial approach to determine if nucleoside transport activity in K562/4 cells changed with hemin treatment, NBMPR-binding activity was examined in K562/4 cells treated with hemin for 3 or 8 days, at which times over 70% of cells were benzidine-positive. Results of one

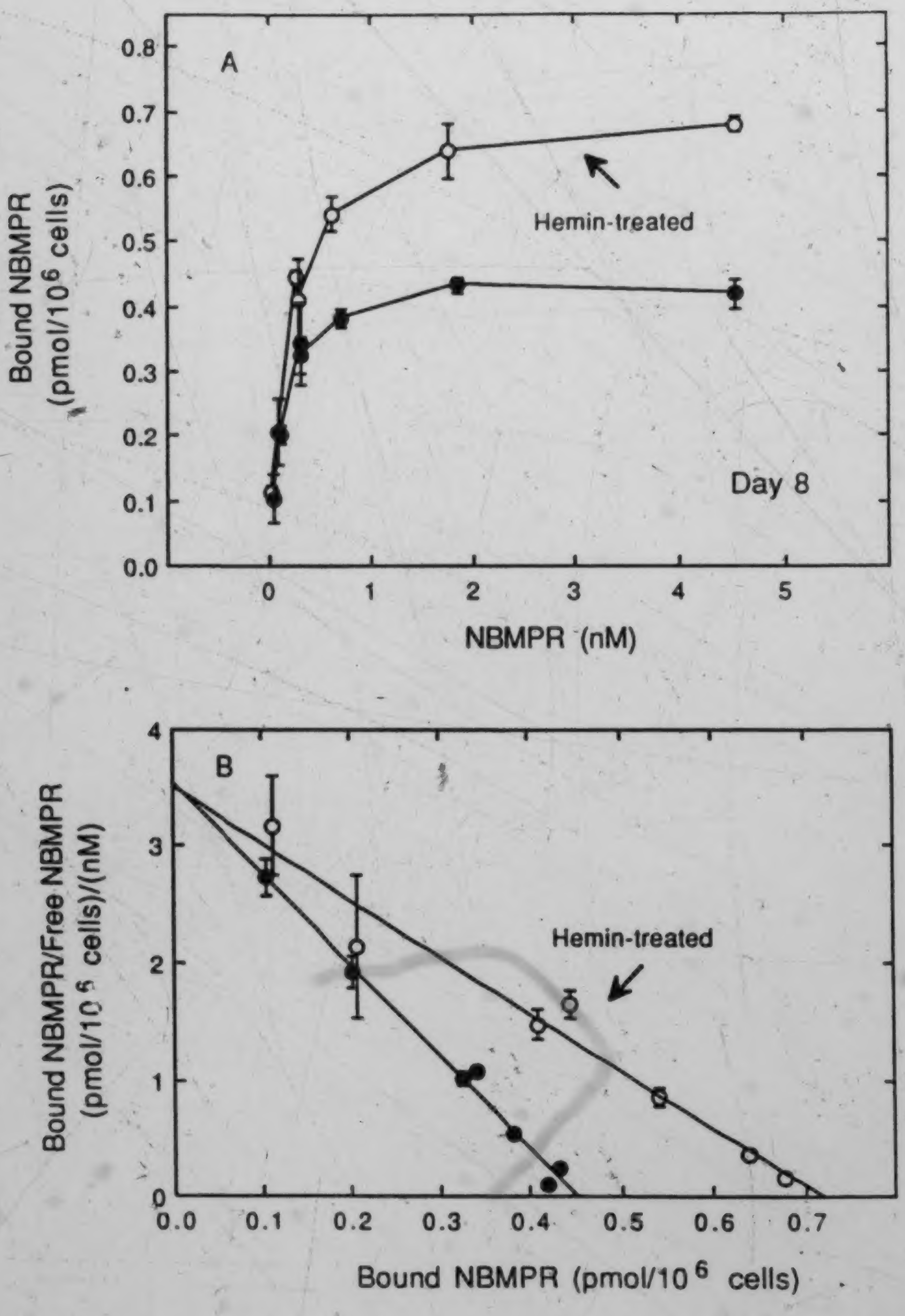
Figure 28

Effect of hemin treatment on site-specific binding of ^{3}H-NBMPR to K562/4 cells

Site-specific binding of ^{3}H-NBMPR was assessed at 22°C under equilibrium conditions as described in Figure 5, using K562/4 cells grown in the presence (open circles) or absence (closed circles) of 50 µM hemin for 8 days. The majority (86%) of cells in the hemin-treated cultures expressed detectable amounts of hemoglobin on the day of the experiment as assessed by the benzidine oxidation test. Binding parameters (see Table VI) were obtained by mass law analysis of NBMPR-binding data. The values are means of triplicate determinations and error bars (S.D.) are shown only where deviations were large enough to extend beyond the symbols.

Panel A: Equilibrium binding of ^{3}H-NBMPR. For clarity, only specifically bound NBMPR is shown. It was calculated by subtracting values for non-specific binding (determined in the presence of 10 µM NBMPR) from those for total binding.

Panel B: Scatchard analysis of the values for specifically bound NBMPR shown in panel A.


A
Bound NBMPR
(pmol/10^6 cells)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
1
2
3
4
5
NBMPR (nM)
Hemin-treated
Day 8
B
Bound NBMPR/Free NBMPR
(pmol/10^6 cells)/(nM)
0
1
2
3
4
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Bound NBMPR (pmol/10^6 cells)
Hemin-treated

such study with hemin-treated and control K562/4 cells are shown in Figure 29. Site-specific binding of NBMPR was saturable in hemin treated cells as in control K562/4 cells (28A) and the Kd values were 0.21 nM and 0.13 nM, respectively (Figure 28B). There was almost a 2-fold difference in binding capacity between hemin-treated and untreated cells, with Bmax values of 4.3 x 10^5 molecules/cell and 2.8 x 10^5 molecules/cell, respectively. After 8 days exposure to hemin, the viability of hemin-treated cells was 90-94% and the majority (86%) were producing hemoglobin.

Results from a series of experiments (including those of Figure 28) in which NBMPR-binding affinities (Kd values) and capacities (Bmax values) were determined after 3 and 8 days of exposure to hemin are summarized in Table VI. For each of the three experiments, Bmax values were greater in hemin-treated cells than in untreated cells grown in parallel, whereas the Kd values were essentially the same. Although the Bmax values for both hemin-treated and untreated cells were within the range of values (4.8 ± 0.9 x 10^5 sites/cell) obtained in earlier experiments (n=9) with untreated cells, when results from parallel cultures were compared, the hemin-treated cells consistently exhibited more NBMPR sites than untreated cells.

Thus, exposure of K562/4 cells to hemin resulted in a small, but reproducible, increase in NBMPR-binding capacity. This suggested that induction of erythroid differentiation of K562/4 cells was accompanied by an increase in nucleoside transport capacity by the NBMPR-sensitive route.

Table V

NBMPR-binding activity in K562/4 cells treated with hemin

Scatchard analysis of equilibrium binding of ^{3}H-NBMPR at 22°C was conducted as described in Figure 6. K562/4 cells were grown in the presence or absence of 50 µM hemin for 3 or 8 days and the densities of cultures when cells were harvested ranged from 4.5 x 10^5. The % of hemoglobin producing cells was determined by the benzidine oxidation test. Each set of values is from a single experiment conducted with parallel cultures.

	Untreated cells			Hemin-treated cells			% increase in Bmax$^\alpha$
	% Benzidine positive cells	Bmax sites/cell (x10^5)	Kd (nM)	% Benzidine positive cells	Bmax sites/cell (x10^5)	Kd (nM)	
Day 3	1	2.9	0.18	77	3.5	0.17	21
Day 8*	6	2.7	0.13	86	4.3	0.21	59
Day 8	nd&	1.9	0.33	67	3.4	0.34	81

* Results from Figure 29

α % increase in the number of NBMPR-binding sites of hemin-treated cells relative to that of untreated cells.

& Not determined

3) *Effect of hemin treatment on adenosine transport by K562/4 cells*

To determine if hemin treatment also stimulated NBMPR-sensitive transport activity, zero-*trans* influx of 1 µM adenosine was examined in the presence and absence of 1 µM NBMPR. Adenosine was chosen as the test substrate since results of the kinetic studies of Chapter III, Section B.4, had indicated that adenosine was a better substrate than uridine for both transport systems of K562/4 cells. The concentration of 1 µM was chosen because it is very close to physiological adenosine concentrations (0.4-0.6 µM) found in human serum (Fox & Kelley 1974).

Figure 29 shows a representative time course for uptake of 1 µM adenosine by hemin-treated and untreated K562/4 cells. Exposure of cells to 50 µM hemin for 9 days resulted in a slight increase in adenosine transport rates. There was, however, a significant increase in sensitivity to inhibition by 1 µM NBMPR, which was more evident when longer time courses for adenosine uptake were compared (Figure 29 inset). From these results, it appears that hemin treatment resulted in an increase in NBMPR-sensitive transport and a decrease in NBMPR-insensitive transport.

Qualitatively similar results were obtained in three separate experiments performed with cells exposed to 50 µM hemin for different lengths of time (Table VI). Small increases (5-35%) in total transport activity were consistently observed in hemin-treated cells and the increase was greater when the transport assay was performed at 37°C (Table VI). Sensitivity to inhibition by NBMPR increased with hemin treatment. Adenosine transport activity of untreated cells was reduced by 90-95% by 1 µM NBMPR whereas that of hemin-treated

Figure 29

Effect of hemin treatment on adenosine transport by K562/4 cells

K562/4 cells were grown for 9 days in the presence (closed symbols) or absence (open symbols) of 50 µM hemin. Zero-*trans* influx of ^{3}H-adenosine (1 µM, 5.2 µCi/ml) was then determined at 22°C, in the presence (triangles) or absence (circles) of 1 µM NBMPR. Uptake assays were conducted at room temperature by the inhibitor-oil stop method to obtain time courses during 3-sec exposures (full drawing) or 60-sec exposures (inset) to ^{3}H-adenosine. Data plotted are the means of triplicate determinations. The S.D. values (omited for clarity) ranged between 0.5-28% of mean values.

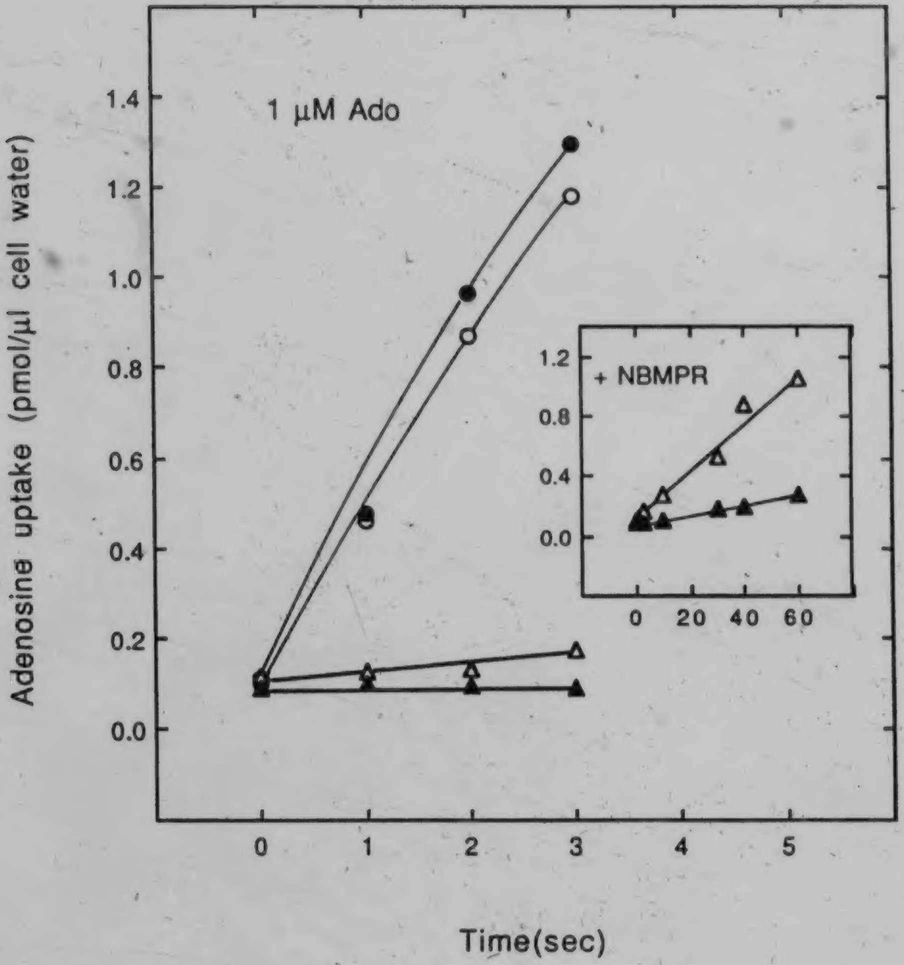

1 μM Ado
Adenosine uptake (pmol/μl cell water)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Time(sec)
+ NBMPR
1.2
0.8
0.4
0.0
0
20
40
60

Table VI

Effect of hemin treatment on 1 μM adenosine zero-*trans* influx by K562/4 cells

The results summarized below were obtained in experiments conducted with cells from the same cultures that were used to estimate NBMPR-binding activity (Table V). Transport rates were derived from parabolic or linear uptake curves fitted to 3-sec time courses (Vt, total influx; Vs, NBMPR-sensitive influx; Vi, NBMPR-insensitive influx). The % of benzidine-positive cells was determined the previous day of the experiment (see Table V).

	Vt	Vs	Vi
		(pmol/μl cell H_2O)sec^{-1}	
Day 4 (22°C; 77% of cells benzidine positive)			
untreated	0.266	0.240	0.025
hemin-treated	0.279	0.274	0.005
(treated/untreated) x 100	105 %	116 %	20 %
Day 9 (22°C; 86% of cells benzidine positive)*			
untreated	0.363	0.346	0.017
treated	0.442	0.439	0.003
(treated/untreated) x 100	122 %	127 %	18%
Day 12 (37°C; 75% of cells benzidine positive)			
untreated	0.920	0.866	0.054
treated	1.248	1.245	0.003
(treated/untreated) x 100	136 %	144 %	6 %

* Results are from experiment in Figure 29

cells was almost completely abolished (98.2-99.8% inhibition). The change in NBMPR sensitivity can be dissected into a small increase in the NBMPR-sensitive transport component and a more pronounced decrease in the NBMPR-insensitive component. The transport rates for the NBMPR-sensitive component increased by 14-26% when transport was measured at room temperature and by about 40% when it was measured at 37°C. These changes were not due to differences in the volume of the cells since the uptake data have been expressed as pmol/μl of intracellular water.

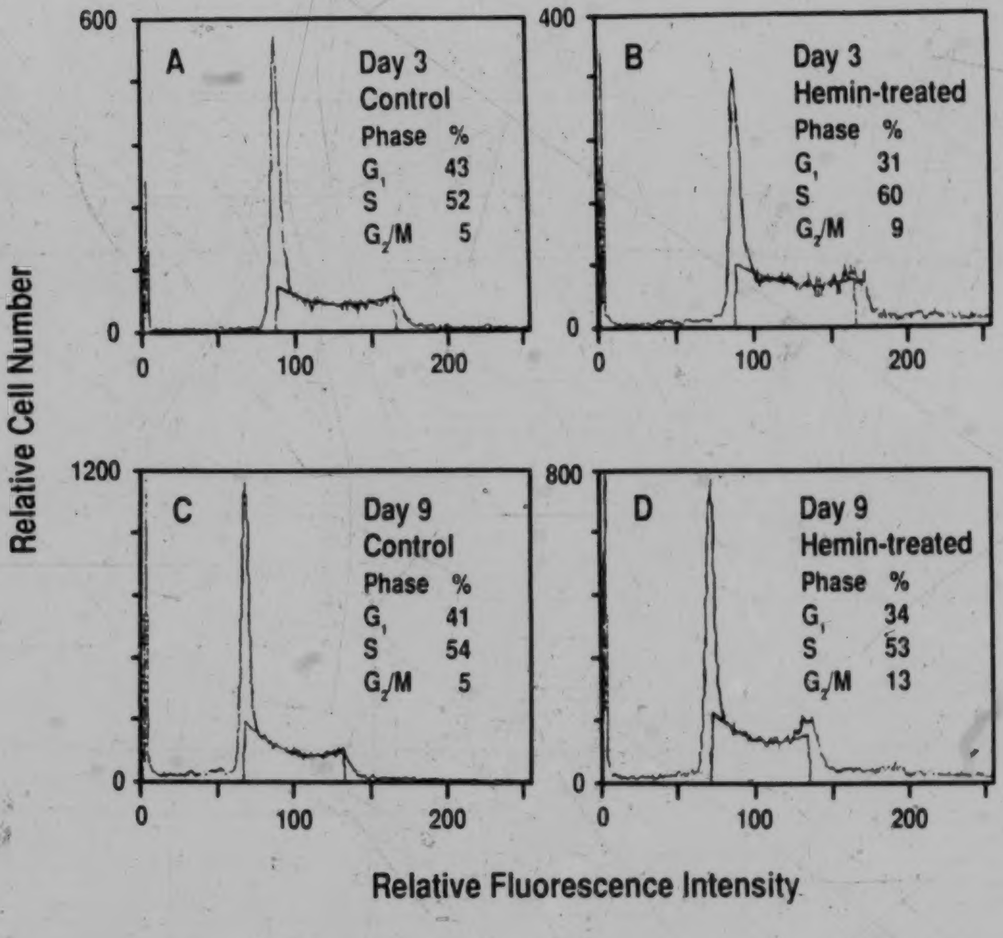
4) *Effect of hemin on cell cycle progression and cell size of K562/4 cells.*

To determine if hemin treatment of K562/4 cells altered the distribution of cells in the cell cycle, flow cytometry was used to analyse DNA distributions in hemin-treated and untreated cells. In Figure 30 are presented histograms of DNA distributions of cells that were cultured for 3 or 9 days in the presence or absence of hemin. Hemin-treated cells continued to proliferate with rates comparable to those of untreated cells and there was no indication of G_1 arrest. There was, however, a small increase (7-12%) in the proportion of cells in the S and G_2/M phases in hemin-treated cultures. The % of benzidine positive cells increased between the 2nd and 9th day of treatment from 33 to 78%. Cell viability was examined daily up to the 6th day of treatment and remained >90%. In a previous report (Sutherland 1984), treatment of K562/4 cells with 25 μM hemin resulted in a 10% increase in the number of G_2/M phase cells.

Figure 30

Effect of hemin treatment on the cell-cycle distribution of K562/4 cells

Cultures (100 ml) of actively proliferating K562/4 cells were initiated at 1 x 10^5 cells/ml and cultured in the absence or presence of 50 µM hemin for 9 days. After the third day, cell population densities were maintained between 3.0 x 10^5 and 7.6 x 10^5 cells/ml by dilution at 48-hr intervals with fresh media. The percentages of benzidine-positive cells on days 3 and 9 were 78% and 45%, respectively. On days 3 and 9, portions of the cultures (about 2 x 10^6 cells) were removed, fixed with ethanol (A, B) or paraformaldehyde (C, D) and stained with propidium iodide as described in Chapter II, Section B6. Cells fixed with ethanol were stored (4°C) for 6 days before they were stained and analysed. Cells fixed with paraformaldehyde were fixed, stained and analysed the same day. The fluorescence intensity of the various treated samples was determined using a Becton Dickinson FACScan fluorescence activated cell analyzer. Results are from a single experiment.


Relative Cell Number
Relative Fluorescence Intensity
A
Day 3
Control
Phase %
G_1 43
S 52
G_2/M 5
600
0
0
100
200
B
Day 3
Hemin-treated
Phase %
G_1 31
S 60
G_2/M 9
400
0
0
100
200
C
Day 9
Control
Phase %
G_1 41
S 54
G_2/M 5
1200
0
0
100
200
D
Day 9
Hemin-treated
Phase %
G_1 34
S 53
G_2/M 13
800
0
0
100
200

Hemin treatment of K562/4 cells also resulted in a small increase in the population of cells with larger volume (data not shown).

In summary, there were small differences in the growth state of hemin-treated and untreated K562/4 cells. With hemin treatment, there was a small decrease in the proportion of cells in the G_1 phase and a corresponding increase of cells in the G_2/M phase of the cell cycle. There was also an increase in the proportion of cells with higher volumes, which was consistent with the presence of more G_2/M cells in hemin-treated cultures, relative to untreated cultures.

C. SUMMARY

When K562/4 cells were exposed to 50 µM hemin, proliferation rates and viability were slightly decreased and the percentage of hemoglobin-producing cells increased from a basal level of 1-5% to as high as 85% after treatment for 2-3 days. There was a progressive increase in the amount of hemoglobin produced, reaching a 15-fold increase after 4 days treatment. Thus, treatment of K562/4 cells with hemin resulted in production of hemoglobin, a response considered to be a marker of erythroid differentiation

Exposure of K562/4 cells to hemin resulted in increases in the number of NBMPR-binding sites (20-80%) and NBMPR-sensitive transport activity (16-44%) and decreases in NBMPR-insensitive transport activity (80-95%). These changes indicate that hemin-induced differentiation of K562/4 cells affected the two transport components differently, with an increase in NBMPR-sensitive transport activity and a decrease in NBMPR-insensitive transport activity.

Cell-cycle analysis by flow cytometry demonstrated that the growth state of K562/4 cells was altered by hemin-treatment. There was an accumulation of K562/4 cells in the S and G_2/M phases and a corresponding increase in the proportion of cells with larger volumes.

CHAPTER VI-DISCUSSION

The main objectives of this work were to determine if K562/4 cells possess an erythrocyte-like nucleoside transport system, to identify and partially characterize membrane protein(s) associated with this transport process and to assess functional and structural similarities between the nucleoside transporter of K562/4 cells and human erythrocytes.

The approach taken to characterize the nucleoside transport phenotype of K562 cells was to study the kinetic properties of nucleoside influx under zero-*trans* conditions and examine the heterogeneity of the process on the basis of sensitivity to inhibition by NBMPR. The kinetic studies were conducted at 37°C to obtain physiologically relevant transport rates.

Influx was measured as the initial rate of uptake of ^{3}H-labelled permeant. The true initial rate of uptake is the rate of cellular accumulation of permeant at theoretical time "0". The slope of the tangent of the uptake progress curve at experimental time "0" gives a good approximation of initial rate. The problems posed when one studies transport at 37°C, because of the rapidity of the transport process, are (i) to determine accurate experimental time "0" values and (ii) to effectively stop the transport reaction once initiated.

In a previous study with cultured L1210 leukemia cells (Hogue et al 1990), a combination of cooling to 4°C and chemical quenching with 100 μM dilazep, followed by separation of cells from the nucleoside-containing medium by rapid centrifugation, was effective in "instantaneously" stopping transport reactions. This procedure was effective in quenching the transport of uridine and adenosine (Figures

2 & 3) in K562/4 cells at 37°C, and allowed measurements of uptake assays within short time intervals ($\leq$ 3sec). This rapid sampling methodology was used to study nucleoside influx without any significant interference by intracellular metabolism or backflux.

Fluxes of uridine, adenosine and thymidine in K562/4 cells were not completely inhibited by NBMPR, as indicated by the biphasic concentration-effect curves (Figure 5). These results suggested at least two processes of nucleoside transport in K562 cells, distinguishable on the basis of their sensitivity to NBMPR. One process was inhibited by nanomolar concentrations of NBMPR and represented 80-90% of total uridine, adenosine and thymidine influx at low (6-10 μM) and high (298-327 μM) concentrations. The IC_{50} values estimated for this route, defined as NBMPR-sensitive, were 0.4-1.0 nM. The other process(es), defined as NBMPR-insensitive, was not totally blocked, even with 5 μM NBMPR, and it was not possible to determine IC_{50} values because of the limited solubility of NBMPR.

K562/4 cells expressed a large number of sites ($B_{max} = 4.8 \pm 0.9 \times 10^5$ cells/ml) to which NBMPR bound with an affinity ($K_d = 0.3 \pm 0.1$ nM) that was within the range ($K_d = 0.1$-1.0 nM) previously observed for erythrocytes and several types of cultured cells (Gati & Paterson 1989a). Inhibition of nucleoside influx by the NBMPR-sensitive route appeared to be due to occupancy of the high-affinity binding sites. The K_d value for site-bound NBMPR at 37°C ($K_d = 0.5$ nM) was similar to the IC_{50} values (0.4 - 1.0 nM) for inhibition of the NBMPR-sensitive process, consistent with the conclusion that occupancy of the high-affinity sites by NBMPR blocked the NBMPR-sensitive transport process.

Nucleoside transport in K562/4 cells had broad substrate specificity and was stereoselective. Purine and pyrimidine nucleosides (uridine, adenosine, thymidine, formycin B) were permeants for both the NBMPR-sensitive and NBMPR-insensitive routes of transport while L-uridine was a poor permeant, even at high concentrations (Figure 19).

Both NBMPR-sensitive and NBMPR-insensitive transport processes appeared to be equilibrative in nature. In studies of uptake of 25 µM formycin-B, in either the absence or presence of NBMPR at a concentration sufficient to saturate the high affinity sites, the transport reaction reached a steady state when the intracellular and extracellular concentrations of formycin B were similar. Entry of adenosine and uridine by the NBMPR-insensitive route did not require the presence of Na^+. These results indicated that, under the conditions used in this study, concentrative transport activity, if present, was minimal. However, results of studies conducted with 50 µM formycin B raised the possibility of a small component of concentrative uptake, which from the results presented here, cannot be ruled out.

The Michaelis-Menten equation and its linear plots, which describe the kinetics of a simple carrier model, were used to derive kinetic parameters for influx of uridine and adenosine. The estimation of fluxes as rates of uptake at time "0" assures that the concentration of the nucleoside that has been transported is much smaller ($< 5\%$ of S_1) than the extracellular nucleoside concentration and that there is, therefore, no backflux. The differential form of the Michaelis-Menten equation and its linear plots can be used to analyze kinetic data without a need for integrated forms of the equation (Segel 1975a). In this work, the rate versus concentration data were analyzed according to three methods to minimize the influence of the statistical errors inherent in the various linear transformations of the Michelis-Menten equation.

Kinetic analyses of NBMPR-sensitive and NBMPR-insensitive components of adenosine and uridine fluxes were undertaken by measuring zero-*trans* fluxes in the absence or presence of 0.5 or 0.1 μM NBMPR and separating the fluxes into two components by subtracting NBMPR-insensitive fluxes from total fluxes. Both components exhibited saturation kinetics. The NBMPR-sensitive process had higher affinity and capacity than the NBMPR-insensitive process, with lower (1/3 to 1/5) K_m values and higher (3-fold) V_{max} values for both uridine and adenosine. Values for the efficiency of permeability (π^{zt}_{12}) were much higher (10-15 fold) for the NBMPR-sensitive system, suggesting that it is the major route of transport at low, physiologically relevant concentrations (<10 μM) of nucleosides. A summary of these results is presented in Table VII.

Differences in substrate affinities for NBMPR-sensitive and NBMPR-insensitive nucleoside transport have also been observed in rat erythrocytes (Jarvis 1986b), chinese hamster ovary cells (Plagemann 1984a), and guinea pig cerebral cortical synaptosomes (Lee 1989). In contrast, results from studies with mouse L1210 leukemia cells (Belt 1983), and rat Walker 256 carcinosarcoma (Plagemann & Wohlhueter 1985a), Novikoff hepatoma cells (Plagemann & Wohlhueter 1984) and cerebral cortical synaptosomes (Lee 1989) indicate that NBMPR-sensitive and NBMPR-insensitive transport systems exhibit similar substrate affinities. All data

Table VII

Summary of kinetic parameters for the NBMPR-sensitive and NBMPR-insensitive processes of K562/4 cells*

	NBMPR-sensitive influx			NBMPR-insensitive influx		
	Km (µM)	Vmax (µM/sec)	π^{zt}_{12} (sec^{-1})	Km (µM)	Vmax (µM/sec)	π^{zt}_{12} (sec^{-1})
uridine	229 ± 39	133 ± 17	0.60 ± 0.17	1077 ± 220	40 ± 5	0.04 ± 0.00
adenosine	61 ± 9	70 ± 5	1.22 ± 0.09	186 ± 31	23 ± 8	0.12 ± 0.03

* Values are means (± S.D.) of results presented in Tables IV and V.

reported in the literature for kinetic parameters of NBMPR-insensitive transport have been obtained at 22°C, and it is known that, in some cell types, Km values for nucleoside transport increase with temperature (Jarvis & Martin 1985, Plagemann & Wohlhueter 1984c). The data obtained in this study were obtained at 37°C, which could account for the higher Km values observed for both uridine and adenosine, as compared to those reported in the literature.

The results of the kinetic studies indicate that nucleoside transport in K562/4 cells is mediated by two facilitated diffusion systems: an NBMPR-sensitive process with high affinity and high capacity, and an NBMPR-insensitive process with low affinity and low capacity. Both processes accepted purine and pyrimidine nucleosides as permeants, indicating broad substrate specificity, and both exhibited greater affinity for adenosine than for uridine. The NBMPR-sensitive process was the major route of transport, particularly at low permeant concentrations.

Although transport by the NBMPR-insensitive process in K562/4 cells appeared to be insignificant for salvaging nucleosides at physiological concentrations, it may be of pharmacological importance. NBMPR did not have a significant protective effect against the antiproliferative action of tubercidin in K562/4 cells (Figure 21). K562/4 cells remained highly sensitive to tubercidin, despite the presence of NBMPR concentrations that were sufficient to completely inhibit the NBMPR-sensitive transport process. Tubercidin evidently accumulated intracellularly at toxic levels through uptake by the NBMPR-insensitive process. NBMPR has been used successfully to protect cells with NBMPR-sensitive transport systems from cytotoxic nucleosides (Paterson et al 1983). Cells with NBMPR-insensitive processes are more susceptible to toxic nucleosides in the presence of NBMPR than cells with NBMPR-sensitive processes.

The reason for the presence of two equilibrative nucleoside transport systems with different affinities in K562/4 cells is not apparent. Expression of the NBMPR-insensitive transport process may be the result of the leukemic origin of K562 cells, or of long-term *in vitro* culture of the cell line. Normal hematopoietic progenitor cells, including erythroid progenitors, seem to possess only the NBMPR-sensitive process, since they can be protected from tubercidin toxicity by relatively low concetrations (0.1-0.3 μM) of NBMPR. For example, a 15-25 fold increase in the IC_{50} for inhibition of colony formation was observed when cells were pretreated with NBMPR before they were exposed to tubercidin (Janowska & Cass 1987).

The relationship between NBMPR-sensitive and NBMPR-insensitive nucleoside transport is not known. Only a few cell types (e.g., human erythrocytes, cultured human RPMI 6410 lymphoma cells, cultured mouse S49 lymphoma cells) are known to express primarily NBMPR-sensitive transport (Paterson et al 1987). In most other cell types studied, NBMPR-sensitive and NBMPR-insensitive transport systems co-exist. It has been speculated that they either represent different functional states of the same protein (Plagemann & Wohlhueter 1985) or products of independent, but closely related genes (Gati & Paterson 1989a). Evidence exists that the two systems can be genetically separated. A mutant of the L1210 mouse leukemia cell line that has lost the equilibrative NBMPR-insensitive but not NBMPR-sensitive activity has been selected (Crawford et al 1990), suggesting that the two equilibrative transporters are products of independent genes.

Comparison of the NBMPR-sensitive nucleoside transporters in K562 cells and human erythrocytes

i) *Functional and kinetic characteristics.*

NBMPR-sensitive nucleoside transport in both K562/4 cells and human erythrocytes is equilibrative and of broad substrate specificity (Cass & Paterson 1972, Plagemann et al 1990) with respect to the base moiety of nucleosides. Both processes are stereoselective with respect to the enantiomeric configuration of the ribosyl moiety and D-nucleosides are the preferred substrates (Gati et al 1989b). Values for Km and Vmax at 37°C for zero-*trans* influx of adenosine and uridine in K562/4 cells (this study) and erythrocytes (Plageman & Wohlhueter 1984b, Paterson et al 1984) are presented in Table VIII, where it is evident that the two cell types exhibit similar kinetic properties, at least for adenosine.

Both K562/4 cells and human erythrocytes express binding sites with high affinity for NBMPR. Occupancy of these sites by NBMPR results in inhibition of transport in K562 cells (this study) and in erythrocytes (Cass et al 1974). K562/4 cells express 50-fold more NBMPR-binding sites than human erythrocytes. Since the volume of K562/4 cells differs greatly from that estimated for human erythrocytes, it is more appropriate to compare the surface density of NBMPR-binding sites than the total number of sites. A rough estimate of surface binding-site densities can be derived if one assumes (i) a spherical shape for cells, with estimated volumes of 2.0 ± 0.3 x 10^{-6} µl for K562/4 cells (this study) and 0.17 ± 0.06 x 10^{-6} (Steck 1974) and (ii) a surface location for the binding sites, estimated to be 4.8 x 10^5 for K562/4 cells (this study) and 1.1 x 10^4 sites/cell for erythrocytes (Jarvis 1983). Using these figures, K562 cells (r=7.8 µm, A=764.2 μm^2) express 628 sites/μm^2 and erythocytes (r=3.4 µm, A= 145.2 μm^2) express 80 sites/μm^2.

Despite the 8-fold higher apparent surface density of NBMPR-binding sites, the catalytic efficiency of the NBMPR-sensitive transporter of K562/4 cells was much lower than that of erythrocytes. The translocation capacity (Vmax/Bmax) of the two cell types differed by 5-fold, suggesting either that the erythrocytic transporter is considerably more efficient or, more likely, that not all of the NBMPR-binding sites of K562/4 cells represent functional transporters. Similar differences between the transport capacities of erythrocytes and other nucleated cell types have been observed previously (Young et al 1983, Plagemann 1985), and it has been suggested that some of the transporters quantitated by high-affinity binding of NBMPR in nucleated cells may be non-functional, perhaps because they are associated with internal membranes. Because of its high lipid solubility, NBMPR equilibrates across membranes within a few sec and internal transporter elements would be readily accessible to NBMPR under the conditions used to measure equilibrium binding.

In conclusion, the NBMPR-sensitive nucleoside transporter of K562 cells appears to have kinetic properties that are similar to those of the nucleoside transporter of human erythrocytes.

Table IIX

Comparison of NBMPR-sensitive nucleoside transport characteristics and some other features of K562 cells and human erythrocytes

	K562/4 cells	Human erythrocyte
Km (μM)		
Uridine	229 ± 39	141 [a]
Adenosine	61 ± 9	98 ± 17 [a]
Vmax (μM/sec)		
Uridine	133 ± 17	175 [a]
Adenosine	70 ± 5	80 ± 9 [a]
Bmax		
NBMPR-binding sites/cell (x 10^5)	4.8 ± 0.9	0.11 ± 0.01[b]
Kd (nM)	0.3 ± 0.1	0.31 ± 0.02 [b]
Cell volume (x 10^{-6} μl)	2.0 ± 0.3	0.171[c]
Vmax/Bmax		
(molecules/site/sec)		
Uridine	334	1640
Adenosine	175	750
Surface density of NBMPR sites		
(NBMPR sites/μm^2)	628	80

[a] Data obtained at 37°C (Plageman, 1984, Paterson A.P.R et al., 1984)

[b] For fresh erythrocytes (Jarvis et al 1983).

[c] Taken from Steck T.L. (1974)

Both transporters bind NBMPR equally tightly and inhibition of transport is associated with saturation of the NBMPR-binding sites. These similarities in functional properties suggested that K562/4 cells express an erythrocyte-like NBMPR-sensitive nucleoside transporter.

ii) *Molecular properties*

K562/4 cells expressed NBMPR-binding polypeptides that were identified by covalent cross-linking of ^{3}H-NBMPR to membranes upon exposure to uv light (Figure 21). The ^{3}H-labelled material migrated in SDS-electropherograms as a single broad band (mean peak molecular mass 63 ± 3 kDa). The specificity of photolabelling was shown by the disappearence of this band when excess non-labelled NBMPR was present in reaction mixtures. The average molecular mass of the K562/4 radioactive peak was 10-12 kDa greater than that of the human erythrocyte, suggesting structural differences in the NBMPR-binding polypeptides of the two cell types.

The electrophoretic mobilities of the K562/4 and erythrocyte polypeptides were compared after treatment of the ^{3}H-NBMPR-labelled membranes with N-glycosidase F (Figure 24). The migration profiles of both polypeptides showed sharpening and the material moved faster in SDS-polyacrylamide gels after deglycosylation. The ^{3}H-peaks for the K562/4 and erythrocyte polypeptides migrated with apparent molecular masses of 42 ± 2 kDa and 44 ± 2 kDa, respectively. These results indicate that the difference in the molecular mass between the K562/4 and erythrocyte NBMPR-binding polypeptides was primarily due to greater glycosylation of the K562/4 polypeptides.

Proteolytic digestion of the K562/4 and erythrocyte polypeptides into smaller fragments suggested differences in primary structure (Figures 25 and 26). When membranes were treated with *Staphylococcus aureous* V8 protease, the ^{3}H-material of K562/4 cells migrated diffusely in the region of 22-58 kDa, whereas that of erythrocytes migrated as three bands (average molecular masses of 35, 25 and 22 kDa). In a previous study, when erythrocyte membranes were subjected to V8 digestion, the ^{3}H-NBMPR-labelled fragments had average molecular masses of 35, 28 and 16 kDa (Klip and Walker 1986).

Thus, the NBMPR-binding polypeptide of K562/4 cells is a glycoprotein that is larger in size than the erythrocyte nucleoside transporter, apparently because of greater glycosylation. This difference in glycosylation did not seem to affect the function of the protein since the kinetics of NBMPR-sensitive transport were relatively similar in both K562/4 cells and erythrocytes. This is consistent with results of a study with L1210 cells in which it was demonstrated that deglycosylation of membrane proteins did not alter NBMPR-binding activity or NBMPR-sensitive uridine transport activity (Hogue et al 1990).

Peptide mapping by limited proteolysis with V8 protease suggested differences in the primary structure of K562/4 and erythrocyte proteins. However, these differences, may not be real. Heterogeneously glycosylated peptides of the same molecular weight would run with different mobilities in SDS PAGE.resulting in differences in peptide mapping analysis.

The possibility exists that the K562/4 nucleoside transporter is a different isoform than the erythrocyte protein, as has been demonstrated for the glucose transporter of human HepG2 hepatoma cells and normal hepatocytes. HepG2 cells express exclusively the "erythroid/brain" glucose transporter isoform while normal hepatocytes express the "liver" isoform. The HepG2 glucose transporter has only 55% homology with the hepatocyte glucose transporter (Thorens et al 1988) and the two isoforms differ in their function. The "liver" glucose transporter has a higher Km for glucose, (15-20 mM) than the "erythroid/brain" glucose transporter (1-2 mM) (Wheeler & Hinkle 1985).

Changes in nucleoside transport upon hemin treatment.

The objective of the work presented in Chapter V was to determine if induction of K562/4 cells to erythroid differentiation resulted in changes in nucleoside transport activity. Although the data are preliminary, they are presented here as a basis for further studies.

Hemin was chosen to induce erythroid differentiation in K562 cells because of its suggested role in maturation of normal erythroid cells (Bonanou-Tsedaki et al 1981, Porter et al 1979). Hemin induces hemoglobin accumulation in K562 cells and enhances expression of other erythroid markers (Benz et al 1980, Rutherford et al 1979, Charnane & Maniatis 1979). However, the precise mechanism by which hemin modulates differentiation in K562 cells has not been established and it is known that exposure of K562 cells to hemin does not trigger commitment to the erythroid phenotype or terminal cell division. When K562 cells are transferred from hemin-containing media to hemin-free media, they show diminishing levels of hemoglobin, which finally reach the levels seen in untreated cells (Dean 1981), indicating that induction of hemoglobin production by hemin is a reversible phenomenon.

In this work, treament of K562/4 cells with 50 µM hemin for 2-3 days resulted in an increase in the proportion of hemoglobin-producing cells, as detected by benzidine staining, from 1-5% to as much as 85%. During a 3-day exposure (Figure 17D), there was a rough correlation between the length of hemin treatment and the increase in cellular hemoglobin content. Hemoglobin production was not related to cessation of proliferation since hemoglobin production and proliferation continued, for periods as long as 11 days, when cultures were diluted with fresh hemin-containing growth media at 48- or 72-hr intervals.

Although hemin treatment resulted in small changes in total adenosine transport activity, the data suggested that the relative contributions of the NBMPR-sensitive and NBMPR-insensitive transport processes differed markedly in hemin-treated and untreated cells. The contribution of the NBMPR-sensitive process increased from 90-95% in untreated cells to 98-99.8% in hemin-treated cells and that of the NBMPR-insensitive process decreased, from 5-10% to 0.2-2.0%. After 11 days treatment with hemin, only the NBMPR-sensitive process was operative in K562 cells (Table VI). Thus, hemin treatment of K526/4 cells appeared to result in up-regulation of NBMPR-sensitive adenosine transport activity and a concommitant down-regulation of NBMPR-insensitive transport activity.

K562/4 cells in hemin-treated cultures were heterogeneous with respect to hemoglobin content. In a single culture, cells exhibited a broad range of staining intensities, indicating different quantities of hemoglobin. If levels of hemoglobin expression are closely related to acquisition of the erythroid phenotype, then the differences observed in benzidine staining of cells in hemin-treated cultures may reflect differences in the maturational stage of cells. If so, differentiation-associated changes in transport activity might be difficult to discern, since they would be diluted in a population of cells that differed in their expression of the differentiated phenotype.

Hemin caused changes in the growth state of K562/4 cells that appear to be more pronounced after prolonged treatment. Analysis of the relative DNA contents of hemin-treated and untreated cells by flow cytometry showed differences in the distribution of cells in the cell cycle, with a small accumulation of cells in the G_2/M phase in hemin-treated cells. It is not clear, even from earlier reports (Sutherland 1984), whether cellular differentiation influenced growth kinetics or whether changes in growth kinetics were due to non-specific actions of hemin.

Erythroid differentiation of Friend murine erythroleukemia cells is accompanied by prolongation of G_1 phase and growth arrest (Marks & Rifkind 1978, Terada et al 1977), suggesting a relationship between the differentiative response and cessation of DNA synthesis. However, in other cell types, the relationship between cessation of DNA synthesis and differentiation depends upon the type of agent used. When various antineoplastic agents were used as inducers, differentiation of human myeloblastic leukemia (ML-1) cells followed inhibition of DNA synthesis and sustained arrest in G_1 phase. In contrast, when 2-O-tetradecanoylphorbol-13-acetate was used to induce differentiation, phenotypic changes occured before blocking of DNA synthesis and there was an accumulation of cells in the G_2/M-phase as well as the G_1 phase of the cell cycle.

Although the relationship between the growth state and the transport activity of K562/4 cells is not clear, the increase in transport activity could have been a reflection of the presence of more G_2/M cells in hemin treated cultures. If so, the changes in transport rates were not due to increases in the cell volume since rates were expressed as pmol/µl cell water/sec.

When human HL-60 promyelocytic leukemia cells were serum starved to stop growth, the number of specific binding sites for NBMPR was reduced by 75% after 24 hr (Stoeckler et al 1988), suggesting that cessation of proliferation was accompanied by a decrease of NBMPR-sensitive transport elements. A reduction in araC transport, observed when HL-60 cells were induced to differentiate by treatment with retinoic acid, appeared to be due, at least in part, to altered cell cycle distribution (Takimoto et al 1989), although it is not clear if these results represent real changes in transport since fluxes were expressed as pmol/10^6 cells/sec.

Results from two studies, which examined NBMPR-binding capacity and nucleoside transport activity as a function of growth state, suggest that changes in nucleoside transport function do not necessarily accompany changes in growth state. In one study (Cass et al 1987), an increase in the total number of NBMPR sites (2- to 3- fold) was demonstrated as HeLa cells progressed from the G_1 phase to the S and G_2/M phases of the cell cycle, but when changes in cell mass were taken into consideration, the observed increase in binding capacity was insignificant. The kinetic parameters of adenosine transport, which were determined at mid-G_1 and mid-S phase, were also similar, and the relative proportions of NBMPR-sensitive and -insensitive adenosine transport activity remained constant throughout the cell cycle. These results were interpreted as indicating tight regulation of production of these two transporters as cells grow in preparation for cell division (Cass et al 1987). In the other study (Plagemann & Wohlhueter 1985a), the proportion of NBMPR-sensitive and -insensitive uridine transport activities in P388 mouse leukemia cells remained constant, despite changes in growth state.

This preliminary study demonstrated changes in nucleoside transport function during hemin induction of erythroid characteristics in K562/4 cells. Perhaps the most interesting observation was the almost complete disappearence of NBMPR-insensitive transport activity, since mature human erythrocytes apparently lack the NBMPR-insensitive transporter. Whether the changes observed in hemin-treated K562/4 cells were due to acquisition of a differentiated phenotype or to non-specific effects of hemin is uncertain. In a brief report (Stoeckler et al 1988), K562 cells exhibited a 2- to 3-fold increase in NBMPR-binding sites after exposure to 1 mM sodium butyrate, which induced hemoglobin synthesis and slowed proliferation by 50%. Growth arrest by exposure to 5'-methylthioadenosine was not accompanied by changes in NBMPR-binding activity, suggesting that the changes induced by treatment with sodium butyrate were not simply due to inhibition of proliferation.

Because the changes observed in nucleoside transport during hemin-treatment of K562 cells were small, it would be difficult, under the conditions used in this study, to study mechanisms responsible for changes in transport function. It appeared that the changes in transport activity were more pronounced with prolonged hemin treatment, and, before undertaking studies of regulation, the nucleoside transport processes of K562 cells should be examined after longer exposures to hemin. In this study, there was a progressive increase in the hemoglobin content in hemin-treated cells during 4-day exposures and in another study (Dean et al 1981) during 14-day exposures. For the Friend murine erythroleukemia line, accumulation of cells committed to terminal erythroid differentiation is linearly related to the time of exposure to inducer. The nature and concentration of inducer and the genetic history of the cell line have been suggested as factors affecting the probability that a cell will enter the differentiation program (Marks & Rifkind 1978).

Commitment to erythroid differentiation involves events other than accumulation of globin mRNA and hemoglobin synthesis, and it is possible that the use of hemin in this study to induce erythroid differentiation in K562 cells did not favor commitment to terminal differentiation. Hemin does not induce terminal differentiation in Friend cells either, and it has been suggested (Marks & Rifkind 1978) that the various types of erythroid-inducing agents have different primary sites of action, and thus different consequences, in initiating the multistep process of erythroid differentiation.

Other differentiation inducers, or combinations thereof, could be used to induce erythroid differentiation in K562 cells. Two examples of drugs that have been shown to induce hemoglobin expression, or to enhance the inducing effects of hemin, are herbimycin A and doxorubicin (Honna et al 1989, Toffoli et al 1989). Both agents induce high levels of hemoglobin production, and the effects of doxorubicin on erytroid differentiation are irreversible, upon removal of the inducer. In such studies, transport changes should be related to levels of hemoglobin production and cell cycle kinetics. The relative cellular content of hemoglobin could be used as a marker of erythroid differentiation and the content of DNA as a marker of growth state, using flow cytometry for analysis.

In summary, this study demonstrated that nucleoside movement across the plasma membrane of K562/4 cells is mediated by two facilitated diffusion systems. An NBMPR-sensitive, high-affinity high-capacity process and an NBMPR-insensitive, low-affinity low-capacity process. The kinetic properties of the NBMPR-sensitive process are similar with those of the nucleoside transport process in human erythrocytes. Structural comparison of the NBMPR-sensitive nucleoside transporters from K562/4 cells and human erythrocytes revealed a 10-12 kDa difference in molecular mass which was demonstrated to be due to heavier glycosylation of the K562 transporter. Further differences in the primary structure of the two proteins were suggested from peptide map analysis.

Treatment of K562/4 cells with hemin, which induced partial erythroid differentiation, resulted in small changes in adenosine transport activity, mainly characterized by the almost complete disappearence of NBMPR-insensitive activity and an increase in the NBMPR-sensitive activity. Although these results were not conclusive it seems that these changes were not due to growth state related regulation of nucleoside transport but rather to a specific effect of hemin.

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